Studies on the chemical control of Fusarium ear blight of winter wheat (*Triticum aestivum* L.)

Thesis

STUDIES ON THE CHEMICAL CONTROL OF FUSARIUM EAR BLIGHT OF WINTER WHEAT (*TRITICUM AESTIVUM* L.)

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ABSTRACT

The fungicides prochloraz and tebuconazole (at concentrations of 2 μg ml^{-1}) were shown to reduce the mycelial growth of *Fusarium culmorum, F. avenaceum, F. poae, F. graminearum* and *Microdochium nivale in vitro* by over 90 % compared to the untreated control. In addition, chlorothalonil inhibited spore germination of all species and pyrimethanil reduced the mycelial growth of *M. nivale* by over 60 % at 2 μg ml^{-1}, although it was ineffective against the other species. In the glasshouse, prochloraz and tebuconazole were moderately effective in reducing the severity of fusarium ear blight (FEB) caused by *F. culmorum* and *M. nivale*. The fungicides gave less effective control of FEB in the field. There was a significant relationship between the incidence and severity of FEB in 1995 but there was no significant relationship between ear blight and yield in either 1995 or 1996.

It was proposed that the interactions between saprophytic microflora and ear blight pathogens may account for the poor performance of fungicides against FEB *in vivo*. Glasshouse and laboratory studies showed that *Alternaria alternata, Botrytis cinerea* and *Cladosporium herbarum* reduced the severity of FEB caused by *F. culmorum* and this antagonism was attributable to both volatile and non-volatile antibiotic production. The saprophytic species showed inherent variability in their sensitivity to the fungicides tested *in vitro* and in the glasshouse. It was shown that certain fungicides (e.g. pyrimethanil) which reduced mycelial growth of the saprophytic species *in vitro* allowed the pathogen to grow by reducing the antagonism of the microflora species against the pathogen. This may not be true for all fungicides in practice.

It was also proposed that the inefficacy of fungicides to control FEB was due to a failure of the fungicide to reach the site of infection. It was shown, using a fluorescent tracer that retention

of spray was influenced by cultivar, time of application and fungicide. The amount of tracer retained was significantly correlated with the number of extruded anthers of wheat. When radio-labelled prochloraz was applied to the ears of wheat, the prochloraz was retained predominantly on the outer glumes, with very small amounts being retained by the florets and rachis. There was no movement of prochloraz between tissues and the half-life of the active ingredient was 48 hours.

This work illustrates the efficacy of fungicides against *Fusarium* spp. and *Microdochium nivale in vitro*, under glasshouse conditions and in the field, and provides some evidence to explain their poor performance. It is proposed that future work should investigate environmental and biological factors which contribute to ear blight epidemics, in order that a forecasting system for fungicide application can be devised. Also, studies of fungicide activity against antagonistic ear microflora species and studies of fungicide retention and penetration may help to optimise fungicide application to control this disease.

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CHAPTER 1

Introduction and Literature Review

Introduction

Fusarium species cause diseases in most genera of cultivated plants. Economically important diseases include vascular wilt of tomatoes caused by *Fusarium oxysporum f.sp. lycopersici;* vascular wilt of cotton caused by *F. oxysporum f.sp. cubense*; stalk rot of sorghum and corn caused by *F. moniliforme* and vascular wilts of corms, bulbs and tubers of beans, peanuts and soybean caused by *F. solani* (Agrios, 1988). Examples of *Fusarium* diseases of the Graminaceae include hypertrophy of shoots due to production of gibberellins in rice caused by *Fusarium moniliforme (Gibberella fijikuroi)* (Booth, 1971) and *Fusarium* patch of turf grass caused by *Microdochium nivale [Monographella nivalis*, formerly classified as *F. nivale* (Mueller, 1977)] (Smiley *et al.*,1992). Ear rot is a serious disease of maize caused predominately by *F. graminearum* (*Gibberella zeae*) and has been well documented by Sutton (1982). This literature review is confined to *Fusarium* ear blight of small grain cereals, including wheat, barley, oats, rye and triticale.

Symptoms of *Fusarium* Ear Blight (FEB)

Fusarium ear blight symptoms have been described in considerable detail by Atanasoff (1920), following studies of *Fusarium graminearum* infections of wheat. The first sign of blight is a pale brown water-soaked lesion on the glumes which spreads to the site of attachment of the glume to the rachis and beyond until the entire ear is infected. As the ear dries out, the glumes lose their water-soaked appearance and take on the premature colouration of a ripe head. Infection of the rachis leads to blighting of the whole ear, above the point of infection, which may, according to Atanasoff (1920), be due to restricted transport of water and nutrients to the ear, although this has not been confirmed experimentally.

At the advanced stage of disease development, the point of infection on the rachis may be

covered with a cottony, pinkish mycelial growth of the fungus, which, if prolonged warm, moist weather persists (which is stated but not defined by Atanasoff (1920) and may differ from region to region and certainly from country to country), extends over the infected ear and becomes the substratum for conidial development. The conidial masses become dark salmon to grenadine in colour and appear more dense in infections caused by *F. avenaceum* than in those caused by *F. graminearum*. Under dry conditions, conidial production is restricted to an area of infection normally at the base of the spikelet (Atanasoff, 1920). Characteristic symptoms of Fusarium ear blight are shown in Figure 1.

The infected grains are often misshapen or shrunken, failing to develop normally. Observations in USA, showed that the grain of barley may show a grey / brown discolouration with a floury interior (Mathre,1982). Also, under prolonged warm, moist weather ('warm' and 'moist' were not defined), Mathre also observed the presence of blue black perithecia of *Gibberella zeae* (the perfect stage of *F. graminearum)* which had formed on barley ears and which may give the infected ears a characteristic scabbed or speckled appearance. Severe ear blight results in the development of 'tombstone' kernels in the grain; grains are smaller, shrivelled and white to pale pink as observed by Abramson *et al.* (1987), as opposed to grenadine observed by Atanasoff (1920).

It has been suggested that symptoms of FEB on wheat caused by *F. poae* on wheat are distinct from those caused by the other *Fusarium* species. Polley *et al.* (1991b) described them as lesions with a bleached centre and dark brown margin on the glumes. However, these authors failed to distinguish between symptoms of *F. poae* and *F. culmorum, F. avenaceum* and *M. nivale* infection observed as a result of artificially inoculated glasshouse plants. Also, in debate are the

Figure 1. Characteristic symptoms of bleached spikelets as a result of infection by *Fusarium* spp. on ears of winter wheat.

symptoms caused by *M. nivale*, since Rapilly *et al.* (1973) observed a brown glume spot with a dark brown margin which contrasts with observations made by Cassini (1981) who stated that infection of ears by *M. nivale* could only be detected by a loss in thousand grain weight.

Causal Organisms and Geographical Distribution

Fusarium ear blight is caused by five major species including *F. culmorum, F. avenaceum (Gibberella avenacea), F. graminearum* (*Gibberella zeae*, formerly known as *G. saubinetti*), *F. poae* and *Microdochium nivale*; although a further 12 causal organisms have been associated with the disease (Mesterhazy, 1984). The disease has been recorded worldwide (Parry *et al.*, 1995).

The three species of *Fusarium* which dominate as causal organisms of FEB are *F. graminearum, F. culmorum* and *F. avenaceum*. The international distribution of these species seems to depend on their temperature requirements (Parry *et al.*,1995). For example, *Fusarium graminearum* has optimal growth around 20-25°C (Cook, 1980) and as such is generally dominant in regions of the world including USA, Canada, Australia and Central Europe, which have prolonged spells of hot weather, compared to Northern Europe. In cooler regions with a more maritime climate, such as North-west Europe, *F. culmorum* predominates with *F. poae* and *M. nivale* increasing in importance (Polley *et al.*,1991b). Generally, *F. avenaceum* represents a much smaller proportion of the species' isolated from ears, but is present over a wider range of climatic zones.

Early records of FEB in England were made by Smith (1884) who attributed the disease to the fungus *Fusisporium culmorum*. In surveys reported by Moore (1948), *F. graminearum* was

the predominant causal organism responsible for ear blight in the UK in wheat in 1946 and oats in Wales in 1948. McKay (1957), whilst undertaking a disease survey of oats and wheat in Ireland (1930-1954), found that the most common species causing seedling blight, brown foot rot and ear blight were *F. culmorum* and *M. nivale*, although he also suggested that *F. avenaceum* could also be involved in some cases, although no data was presented to illustrate the percentage of ears infected by these species. This contradicts the reports by Moore (1948) who suggested that *F. graminearum* was the predominant species causing the disease in the UK.

In surveys of wheat grain in England, Scotland and Wales in 1989, Polley *et al.* (1991a) showed 3.96 % of grain infection with *F. poae*, 3.43 % *M. nivale*, 1 % *F. avenaceum* and 5.43 % *F. culmorum*. Similar results were obtained the following year. In Scotland 2.5 % of grain was infected with *F. poae*, 4 % *M. nivale*, 1 % *F. avenaceum* and *F. culmorum* <1 %. Records for *Alternaria sp.*, the causal agent of the disease Blackpoint, showed that 1.3 % of seed showed mild infection in England and Wales and 0.8 % in Scotland.

In North America, Detmers (1892) attributed FEB to *Fusisporium culmorum*. However, many of the early records of scab were attributed to *F. graminearum* and it is generally understood that this is the major pathogen in the USA. In an early review of the disease, Atanasoff (1924) regarded the disease as common throughout the cereal (mainly wheat) growing areas of the USA. Comparable records in Canada showed that the disease on wheat, barley, oats and rye was attributed to single species or a combination of the species *F. avenaceum, F. graminearum, F. culmorum, F. poae* and *F. sporotrichioides* (Gordon, 1959). This indicates the widespread confusion at the time as to the causal organism of the disease and the difficulty surrounding the taxonomy of *Fusarium* spp.

The disease has been reported worldwide, including Scotland (Richardson, 1970), Romania (Tusa *et al.*, 1980), Yugoslavia (Tomasovic, 1989) and Australia (Burgess *et al.*,1981).

There has been much conflicting evidence regarding those *Fusarium* species involved in the disease. This is understandable considering the complex of *Fusarium* species sometimes involved in ear blight. Seventeen species were recorded in Hungary which could cause disease symptoms (Mesterhazy, 1984), however, these species respond differently to environmental conditions such as temperature, humidity and rainfall. After isolating *Fusarium* species from wheat ears and the lowest internodes of diseased tillers in South Hungary between 1970 and 1983, Mesterhazy (1984) agreed with the observations of Moore (1948). He found that *F. graminearum* accounted for over 58 % of the isolates and was prevalent in the ear. A further 26.13 % of isolates obtained (from ears, stalk bases and roots) were *F. culmorum* which dominated in the lower stalk, stalk base and root system. Fifteen other species were isolated, including *F. poae, F. avenaceum* and *M. nivale*, however, the incidence of such species was generally low (<2.5 %).

Pathogenicity Studies

A number of workers have shown that *F. graminearum* and *F. culmorum* are consistently the most pathogenic of the *Fusarium* species which infect cereals. In North Dakota, ears showing 'severe' symptoms had over 50 % of the spikelets showing necrotic lesions compared with 10-20 % of spikelets infected where the incidence of disease was lower (Stack and McMullen, 1985). In some fields, the disease was attributed to the species *F. equiseti, F. acuminatum, F. poae, F. trincinctum* and *F. sporotrichioides.* Pathogenicity tests, where *Triticum turgidum* cv. Edmore was inoculated at mid-anthesis, showed that of 44 isolates tested (of seven species), 24 isolates did not cause visible symptoms of ear blight (Stack and McMullen, 1985). Plants

inoculated with *F. graminearum* resulted in severe necrosis of ears with 83% of the ear showing necrotic symptoms. Plants inoculated with *F. culmorum* showed necrosis only in the upper half of the ear. *Fusarium culmorum* and *F. graminearum* caused greater disease severity than the other five species isolated.

Later work involving isolation and pathogenicity tests of *Fusarium* species from wheat showing symptoms of ear blight in Minnesota showed that 75 % of *Fusarium* isolates collected from spring wheat were *F. graminearum* and 17 % were *F. poae*. (Wilcoxson *et al.*, 1988). Thirteen other species were isolated; *F. equiseti, F. sporotrichioides, F. acuminatum, F. oxysporum, F. culmorum, F. moniliforme, F. avenaceum, F. subglutinans, F. sambucinum, F. tricinctum, F. semitectum, F. proliferatum* and *F. crookwellense*, each of which accounted for 1-2 % of the total number of isolations. When isolates of these species were used to inoculate wheat under glasshouse conditions, *F. graminearum* caused all spikelets to become infected compared with 70 % of the spikelets infected by *F. poae*. Details of the conidia concentration were not presented because a mixture of hyphae and conidia was used as opposed to a pure conidial suspension where the number of conidia per ml of water can be determined.

A survey carried out in Eastern Australia by Francis and Burgess (1977) not only revealed *F. graminearum* to be the predominant species responsible for FEB in wheat but also that two distinct sub-populations existed. Isolates which were observed not to readily produce perithecia on media culture or on naturally infected wheat stems were classified as Group I isolates. Those which readily produced perithecia both on naturally infected stems and *in vitro* were classified as Group II isolates. The two groups can be discussed as distinct sub-populations since Group 1 isolates were also frequently associated with crown rot whereas Group 2 produced disease of aerial parts of the plant including FEB.

Spore morphology studies by Gerlach and Nirenberg (1982) also identified two distinct sub-groups of *Microdochium nivale*; *M. nivale* var. *majus* and *M. nivale* var. *nivale*. This was confirmed using a random amplified polymorphic DNA (RAPD) assay by Lees *et al.*, (1995), who whilst studying 48 UK isolates, found that all isolates originating from ears and 72.5 % of isolates originating from stem bases were of the var. *majus* type. Spore morphology studies were also undertaken as a comparison. This represents only a small number of samples, hence further work would help to substantiate these results. It is possible that new molecular tools will assist in future diagnosis of diseases of cereals and in parallel with classical morphology will assist in the classification and identification of *Fusarium* species causing diseases of cereals. For example, Nicholson *et al.*, (1993), whilst using 80 *Fusarium* isolates obtained from ears of wheat showed that restriction fragment length polymorphism (RFLP) analysis using rDNA or total genomic DNA, readily differentiated between the species, *F. avenaceum, F. culmorum, F. lateritium* and *F. poae* and correlated totally with the identification of these species using classic taxonomic procedures.

The Economic Importance of *Fusarium* Ear Blight

Ear blight is of economic importance since it can reduce cereal yields, affect grain quality and affect seed quality.

(i) Effect on Yield of Cereals

Yield of cereals can be divided into three components; (i) the number of stems per unit area (also the number of ears), (ii) the number of grains per ear and (iii) individual grain weight (thousand grain weight). The effect of *Fusarium* infection of cereals has been studied in terms of these three components. Bennett (1933), found that plants inoculated with either *F. culmorum* or *F. avenaceum* at flowering failed to produce grain. Ears inoculated post flowering

produced mycelium-covered rudimentary grain which bore conidia. By inoculating individual ears in a wheat field, a situation approaching natural infection in the field was established with centres of disease within the crop. The disease spread in the direction of the prevailing wind, with the number of affected plants and severity of attack diminishing in proportion to the distance from the centres of infection. The number of grains per ear recorded for plants from uninoculated plots was 57.6 compared to neighbouring ears inoculated with *F. culmorum* which produced only 35.6 grains per ear. For those ears inoculated with *F. avenaceum* only 39.6 grains per ear were recorded. The germination capacities of the samples were also reduced from 89 % in healthy grain to 35 % and 49 % for grain infected with *F. culmorum* and *F. avenaceum*, respectively, suggesting that the use of contaminated seed can have severe effects on the establishment of following cereal crops. These results should be treated with some caution, however, since a small sample size of only ten representative ears were taken for each plot.

Field work in Manitoba using wheat ears inoculated with *F. graminearum* showed that when 28 % of grains were infected, grain yield was reduced by 32 % and thousand grain weight by 34 % (Wong *et al.*, 1992). Although, grain weight per ear was reduced by 36 %, there was no reduction in grain number.

Saur and Benacef (1993) also tried to evaluate the relationship between ear blight symptoms of *F. culmorum* and yield losses in wheat. The severity of disease was evaluated using the number of damaged spikelets at the early dough growth stage and the percentage of visually infected seeds at harvest. Yield and thousand grain weight losses were measured. There was a significant correlation between the number of damaged spikelets and the number of visually infected seeds. There was also an effect due to the variety and year. A linear basis of the yield

loss relationship was provided as a decrease in thousand grain weight as predicted from the number of visually infected seeds rather than the number of infected spikelets. The assessment of visually infected seeds must be very difficult to do, if at all possible, hence the linear basis of the yield loss relationship must be treated with some caution.

Inoculated field trials to examine the resistance of cereal cultivars to FEB have also been used to understand the relationship between disease and yield. For example, Miedaner *et al.* (1993), who worked with resistance in winter rye breeding lines to FEB, found that yield losses of between 27.4 and 48.7 %, resulted from inoculation with *F. culmorum* and 38-51.7 % for *F. graminearum.* In similar work on winter wheat Snijders and Perkowski (1990), found that 1000 grain weight reductions of between 2.8 and 22.4 % were recorded depending on the wheat genotype. This is comparable to yield reductions of between 6.4 and 39.2 % recorded for *F. culmorum* inoculation of 500 wheat lines by Saur, 1991.

(ii) Effect on Grain Quality

Mammalian Toxicity

Mycotoxins are produced by some species of *Fusarium* which contaminate grain. These mycotoxins, including T2 toxin, zearalenone, deoxynivalenol and moniliformin, may pose a threat to animal and human health where the grain is used for animal feed or human consumption. Joffe (1978) reported that the mycotoxin T2 toxin, found in overwintered cereals, was produced by *F. poae* and *F. sporotrichioides* and associated with the development of Alimentary Toxic Aleukia. Long *et al.* (1982), investigated cereal contamination by *F. culmorum* and *F. graminearum,* and found that female pigs fed with this cereal developed vaginal prolapses and vulval vaginitis as a result of the mycotoxin zearalenone. Hoerr *et al.* 1982 reported stunted growth and poor feathering of poultry from feeding with grain infected with

F. poae, *F. culmorum* or *F. graminearum* (Hoerr *et al.*, 1982).

Miller *et al.* (1985) whilst investigating the levels of mycotoxins produced in the grain of the wheat cv. Frederick inoculated at anthesis with *F. graminearum* conidia (5 x 10^3 conidia per ml) and found that both concentrations of Deoxynivalenol (DON) and ergosterol (as a measure of fungal biomass) peaked at 9.5 and 47 μg ml^{-1} respectively, six weeks after inoculation. After this peak the concentrations decreased by 73 and 16 % respectively. These authors concluded that the timing of harvest may influence DON concentration in the crop and suggested that the basis of the decline appeared to be due to the breakdown of DON by plant enzymes. Evidence for this hypothesis of mycotoxin degradation *in vivo* by plant cells was not provided and may have occurred as a result of the extraction process.

Phytotoxicity

Wakulinski (1989) tested the phytotoxicity of six crystalline *Fusarium* mycotoxins; DON, 15-Acetyl-DON, T2 toxin, diacetoxycripenol, moniliformin and zearalenone in wheat seedling tests. All exhibited phytotoxic properties. T2 toxin, DON and 15-Ac-DON showed the most phytotoxic activity at 10-100 times lower concentrations than that of the other toxins. Surface sterilised winter wheat seeds of three genotypes were exposed to the toxins at 1,10 and 100 μg ml^{-1}. After seven days, the percentage of seeds germinated, the weight of the leaves and the weight of the roots were recorded. At 10μg ml^{-1} DON, germination of seed was reduced by 76.6 % when compared to the healthy control for the resistant cultivar Grana and by 65 % in the susceptible variety SMH-684. Leaf weight was reduced by 57.6 % and 35.9 % compared to control weights for these two varieties and root weight by 49.7 and 22 % of the control.

Perkowski *et al.* (1990) analysed the mycotoxins in cereal grain sampled in Poland in 1987

from wheat fields where some heads showed 'fusariosis' symptoms due to natural infection. Grains with visible *Fusarium* damage showed DON concentrations of between 9.6 and 25.3 mg kg^{-1} and an average 3-Ac-DON level of 1.9 mg kg^{-1}. Grains showing no obvious symptoms contained considerably lower concentrations of mycotoxins of between 0.8 and 3.6 mg kg^{-1} for both DON and 3-Ac-DON. The percentage of damaged grains in the sub-samples varied between 16 and 72 %. Bruins *et al.* (1993) studied the phytotoxicity of deoxynivalenol to wheat tissue with regards to *in vitro* selection for Fusarium ear blight resistance. Phytotoxic effects from DON and 3-Ac-DON were tested in seedlings, coleoptile segments and anther derived embryos. Seedling growth response did not differentiate between tolerant and sensitive genotypes according to observed levels of resistance under field conditions. Growth analysis of callus clones failed to show any correlation with known levels of resistance. From these results it was shown that the use of phytotoxicity to mycotoxins had no use for screening for varietal resistance to FEB.

FEB can also affect the bread and beer making qualities of cereals. Berova and Mlanenor (1974), investigated contamination of grain by *F. graminearum* in wheat, and found that the grain protein and gluten were reduced, in addition to the baking quality of the flour. It is, however, unlikely that grain of this poor quality would be passed for baking in the USA and Canada, where standards are set for the contamination of grain by mycotoxins, due to the health risks associated with this level of mycotoxin contamination. In the UK and many other countries, no such standards are set. In brewing, barley grain contaminated with *Fusarium* causes problems of "gushing", that is the uncontrolled foaming of the beer (Narziß *et al.*, 1990).

(iii) Effect on Seed Quality

The contamination of grain with *Fusarium spp.* and *Microdochium nivale* has serious

implications for seed quality since this can provide the primary source of inoculum for the development of seedling blight and foot rot. (Duthie and Hall, 1987). Bechtel *et al.* (1985) using microscopy and polyacrylamide gel electrophoresis, found that invasion of wheat grain by *F. graminearum* resulted in the destruction of starch granules, storage proteins and cell walls. This in turn led to a reduction in seed quality, determined by seed germination and seed vigour tests. Wong *et al.* (1992) recorded a reduction in seed quality when plants were inoculated with *F. graminearum* in the field. There was a reduction in plant emergence from 45.9 % to 34.1 % and grain yield from 2.529 to 2.352 kg per plot.

There may be a need to monitor more closely the contamination of seed by *Fusarium spp* and restrict the use of contaminated seed. During a survey of cereal seed-borne diseases, Reeves and Wray (1994), found that in the seasons 1990 / 1991 and 1993 / 1994, *M. nivale* was the most frequently isolated fungus from wheat seed. Even more disconcerting were results of the 1993 / 1994 seed lot, where 90 % of samples were shown to be infected and of these, more than 90 % failed to meet an arbitrary advisory limit of 5 % seed infection.

Epidemiology of *Fusarium* Diseases on Cereals

Fusarium ear blight occurs as part of a group of diseases of cereals including seedling blight and brown foot rot which occur worldwide (Parry *et al.*, 1994). The epidemiology of the three diseases is inherently linked since many of the species causing ear blight also cause the other diseases. Epidemics of FEB occur sporadically in most cereal growing countries but are normally associated with warm, wet weather during anthesis (Parry *et al.*, 1995). The disease cycle is illustrated in Figure 2.

Fusarium culmorum, F. avenaceum, F. graminearum and *Microdochium nivale* cause seedling

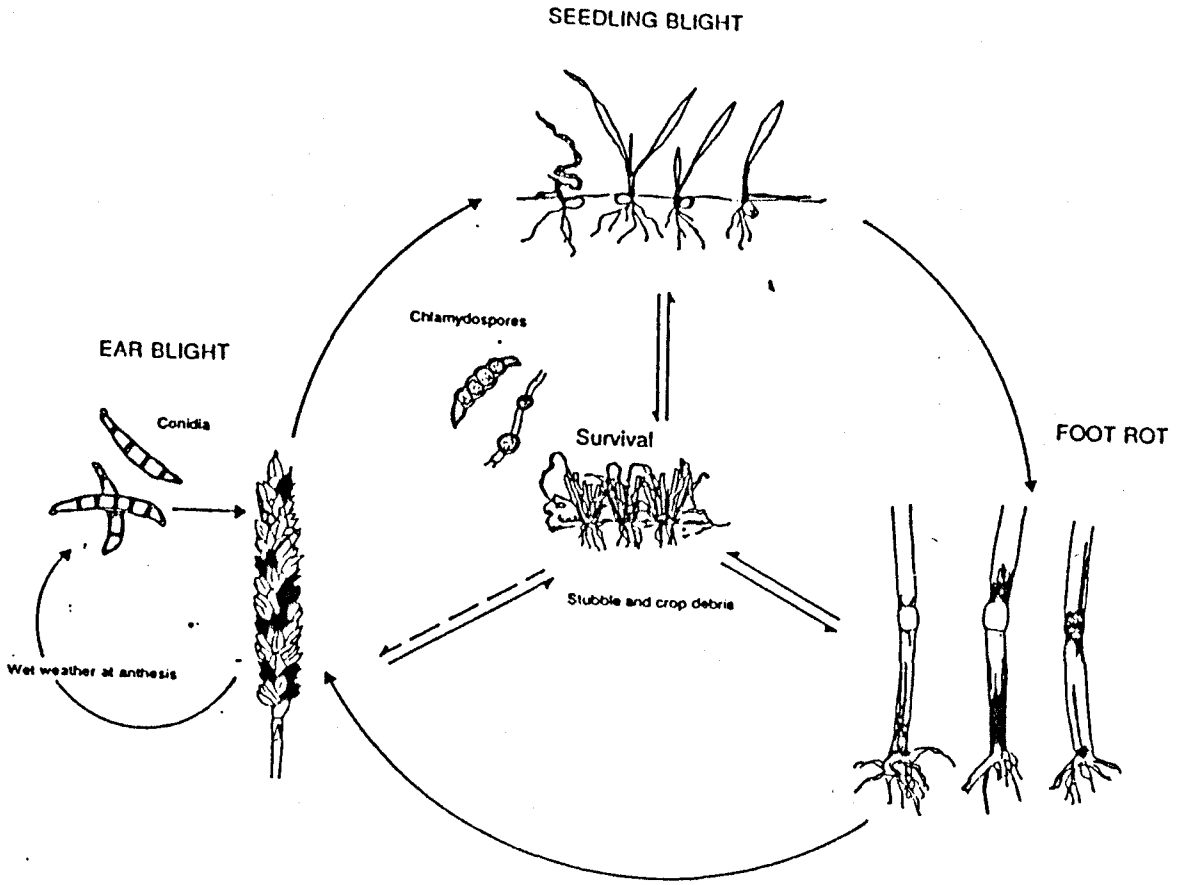


Figure 2. Generalized disease cycle of *Fusarium* on small grained temperate cereals (from Parry *et al.*, 1994)

blight, Fusarium foot rot and Fusarium ear blight of cereals. However, according to Polley *et al.* (1991), *F. poae*, which was commonly isolated from ears in UK surveys, was rarely isolated from plants showing symptoms of seedling blight or foot rot.

Source of inoculum

Perpetuation of the disease cycle depends on the survival of *Fusarium* inoculum which varies for each species. The initial source of inoculum may arise from the soil in the form of chlamydospores or saprophytic mycelium on crop debris (Parry *et al.*,1994). Alternatively, seed and seedlings may become infected as a result of sowing in *Fusarium*-infested soil (Snyder and Nash, 1968) or later in the growing season airborne conidia or ascospores may infect the ears (Francis and Burgess,1977). Infected grain resulting from FEB then provides the inoculum source to complete the disease cycle by causing seedling blight (Cook, 1980).

Sources of inoculum for FEB epidemics may include crop debris. Koehler *et al.* (1924), for example, studied the severity of FEB caused by *F. graminearum* in relation to the previous crop and found that disease was most severe when wheat was grown immediately after maize. The mean percentage of disease recorded over 9 sites was 43.3 % when wheat followed maize. This compared to 27 % when wheat was sown after rye and 22.7 % when sown after oats. The incidence of infected ears dropped to 11.4 % and 8.3 % when wheat was sown after clover and timothy, respectively. Also, the yield of maize susceptible to root rot caused by *F. graminearum*, was considerably reduced where maize followed badly infected wheat in rotation. The author suggested that the use of crop rotations in which neither wheat directly preceded or followed maize would be highly beneficial to wheat growers in the USA.

Observations made by Koehler *et al.* (1924) were supported by Teich and Nelson (1984) who

also recorded that the severity of *Fusarium* ear blight was lower where wheat was not planted after maize. The mean number of ears with symptoms per 100,000 plants was 36 if the previous crop was maize, compared to 7.2 after small grains and 5.2 after soybeans. This provided further evidence for the role of crop debris as a principal reservoir of inoculum. These workers suggested that burying host debris was a means of reducing the primary inoculum other than chlamydospores which can persist for some years in the soil. This is particularly important when wheat follows maize in rotation.

Teich and Hamilton (1985) demonstrated that wheat grains sown immediately after maize had higher concentrations of the mycotoxin deoxynivalenol compared to grain grown in fields where soybeans, barley or mixed grains had been grown previously. Also ear blight was reduced in wheat planted after maize where maize residues were ploughed down or where seed was treated with carboxin plus thiram (Vitaflo 280, Uniroyal Chemical, Elmira, Ontario).

Seedling Blight

Seedling blight occurs as the result of sowing Fusarium-infected seed and is apparent as browning and rotting of the coleorhiza and coleoptile (Atanasoff, 1920). The permanent roots and the first foliage leaf offer a good medium for establishment of *Fusarium* spp. which then penetrates the tissues of the permanent roots and the first foliage which turn brown and rot. The remains of the grain may be covered by mycelium of a dark carmine red colour. Leaves above the infected part of the seedling are normally yellow to brown, the discolouration beginning at the tips. Atanasoff described the process of seedling blight in great detail, however, experimental evidence to show how infection of seedlings occurs was not presented.

Millar and Colhoun, (1969) studied the epidemiology of *M. nivale* on wheat using artificially

inoculated seed. Disease severity was greatest in cold, dry soil conditions. For example, disease was greatest when the soil moisture was 8.8 % and the temperature was 6.1 °C. The severity of disease caused by *M. nivale* depended on both winter and summer soil conditions, since, severe winters cause seedling death and wet summers encourage rapid spread through an infected crop. In contrast, warmer, drier soils have been shown to be conducive to seedling blights caused by *F. graminearum, F. culmorum* and *F. avenaceum*. (Dickson and Mains, 1930; Colhoun *et al.*,1968).

Foot Rot

Fusarium foot rot is apparent on stem bases of cereals as a browning and rotting of the primary roots, crown and scale leaves (Atanasoff, 1920). On the stems of grown plants, the roots and stem close to the ground may become infected around anthesis (Parry *et al.*, 1994), causing rotting of the tissue and a pink or yellowish-brown colouration. This may lead to an interference with water and nutrient movement causing the plant to wilt and possibly lodge. Indeed, when plants are removed they often break at the point of stem rotting, leaving the roots in the soil. The succulent embryonic tissue just above the nodes is particularly susceptible to attack and conidia are often formed at the nodes.

Rennie *et al.* (1983) and Locke *et al.* (1987) suggested that *M. nivale* was the predominant species causing foot rot in the UK, however, *F. culmorum* and *F. avenaceum* were also isolated. This is in contrast to warmer climates including parts of the Australia, USA and Canada where *F. graminearum* and *F. culmorum* were more commonly isolated from stem bases (Burgess *et al.*, 1981 and Cook, 1980).

Ear Blight

The relationship between *Fusarium* foot rot and ear blight is still unclear. However, foot rot may provide the primary source of ascospore and conidial inoculum (depending on the species) for an ear blight epidemic. Infection of cereal grain by the pathogen then completes the lifecycle, since when infected seed is sown for a new crop, this can provide inoculum for the development of seedling blight and foot rot (Duthie and Hall, 1987).

Epidemiology of *Fusarium* ear Blight

Source of Inoculum

Cook (1980) made the connection that ear blight occurred mainly in humid climates where the primary source of inoculum came from either airborne ascospores (in the case of *F. graminearum*) or water splashed conidia deposited directly in or among spikelets of the ears during flowering. It was speculated that the presence of contaminated stubble and conidia on the stem bases of crop plants causing foot rot symptoms, provides a source of inoculum (Parry *et al.*,1994).

It is possible that other plant genera present a source of inoculum for ear blight, as alternate hosts for the pathogen. Jenkinson and Parry (1994a) isolated 226 *Fusarium* isolates from the stem-bases of 14 out of 15 weed species sampled from fallow land. Of the *Fusarium* species isolated, 50.4 % were *F. avenaceum*, 38.9 % were *F. culmorum*, 7.1 % *F. poae*, 2.5 % *F. sambucinum* and 1.3 % *F. graminearum.* Although none of the weed species showed symptoms of *Fusarium* infection, when 79 of these isolates were used to artificially inoculate seedlings of winter wheat (cultivar Mercia) in pots, 77 were shown to be pathogenic. Of these pathogenic isolates, 33 were *F. avenaceum*, 27 *F. culmorum*, 9 *F. poae*, 5 *F. sambucinum* and 3 *F. graminearum*. This study does not conclusively prove that alternative hosts are the source

for ear blight inoculum, however, it appears that they may have an important role to play. It was speculated that this may be particularly important in the case of *F. poae*, which unlike the other important *Fusarium* species, does not produce chlamydospores in the soil and has rarely been isolated from crop debris. Alternate hosts would, therefore, provide a valuable reservoir of inoculum.

Dispersal of Inoculum

Millar and Colhoun (1969) investigated the spread of *M. nivale* through the crop by studying the spread of aerial inoculum using a 'Rotorod' sampler to trap spores. They found that after rain or simulated rain, the concentration of ascospores increased between 10 and 20 times of that normally found in the atmosphere, reaching a maximum 10 minutes after rain had stopped and returning to normal 40 minutes later. Ascospores were trapped 15 cm above ground but not at ear height. The position of perithecia on the tillers of winter wheat were recorded. For inoculated plants (cultivar Viking), 82 % of plants had perithecia at the base of tillers and 96.4 % had perithecia at the top of the tillers. For control (uninoculated plants) 70 % of plants had perithecia at the base of the tillers and 96.3 % of plants had perithecia at the top of the tillers. It was concluded that aerial infection of the crop had occurred, as a result of a uniform distribution of inoculum in the form of ascospores or conidia, giving a uniform infection of the upper parts of the crop. No demonstration of infection by ascospores (for example, microscope work at the plant surface) accompanied this conclusion, however, perithecia were observed at the time of flowering. When moistened with water the perithecia liberated ascospores in a gelatinous substance which, on drying, released the ascospores into the atmosphere.

Jenkinson and Parry (1994b) provided evidence for the upward dispersal of spores of *F. culmorum* and *F. avenaceum* resulting from impacting raindrops. It was shown using selective

agar strips to collect spores, that spores had the potential to be dispersed from the ground to ears in one movement or as a series of movements from leaf-to leaf up the plant. For *F. culmorum,* spores were recorded up to 60 cm vertically and in excess of 100cm horizontally. For *F. avenaceum,* the distances were up to 45 cm horizontally and 90 cm horizontally. The results are similar to those for pathogens known to be splash dispersed such as *Septoria nodorum* (Griffiths and Ao, 1976) and *Pseudocercosporella herpotrichiodes* (Fitt and Bainbridge, 1983).

Snijders (1990) investigated systemic movement of *F. culmorum* in winter wheat under controlled conditions, where fungal growth could not be attributed to rain splash. Mycelial growth was observed in stem tissue over 65cm from the crown in one wheat genotype and in plants growing in inoculated soil, suggesting that crown rot can lead to infection of the higher stem internodes. However, there was no evidence for systemic fungal growth leading to infected ears.

Arthropod vectors have been suggested as a means of transmitting *Fusarium* inoculum. Cooper (1940) showed that the mite *Siteroptes graminum* acted as a vector of *F. poae.* Sturz and Johnston (1983) also observed a high infestation of barley ears by the thrips *Lemothrips denticornis.* The barley ears happened to have FEB, therefore providing circumstantial evidence of this relationship, although evidence of the thrips acting as vectors was not demonstrated. A similar observation was made in maize. Windels *et al.* (1976) associated *Fusarium* species with picnic beetles on maize ears. Maize ears were buried by tillage operations during the previous Autumn and were randomly selected and removed in July 1972 and 1974. All ears were infested with *Glischrochilus quadrisignatus* at the larval, pupal and adult stages. Almost all buried ears were colonised by *Fusarium* spp. In both years 94 % of ears were colonised by

Fusarium species. Eighty-four percent were colonised by *F. solani*, 25 % by *F. moniliforme* and 12 % by *F. oxysporum*. Grains were frequently colonized by more than one species of *Fusarium*, as observed by isolating the pathogens on pentachloro-nitrobenzene agar.

In South Africa, Kemp *et al.*, (1996) showed that Fusarium glume infections on wheat were consistently associated with the mycophageous mite *Siteroptes avenae*. *Fusarium poae* was isolated from glume lesions. When a suspension of microconidia was fed to mites taken from infected ears and their progeny placed on uninoculated, the symptoms which developed were typical of those observed in the field. It was concluded that the mite acted as a vector and the fungus was responsible for causing *Fusarium* glume spot of wheat in South Africa.

Factors Affecting Infection

In an early review of FEB, Atanasoff (1920) found in natural infections first blight symptoms occurred in the latter part of anthesis, followed by more destructive secondary infections, those infections initiated after the first/primary ones. This led to the conclusion that blossoming (anthesis) was the most susceptible stage of cereal development and it was suggested that spores remained on infected heads until the heads reached a susceptible stage before infection took place.

Detailed observations of ear blight symptoms, as a result of artificial inoculation of ears were made by Bennett (1927). Ears of the winter wheat cv. Rector were artificially inoculated with a conidial suspension of *F. culmorum* or *F. avenaceum* and either left uncovered or covered with glass test-tubes to increase infection, for 3 or 7 days. First inoculation was at flowering when the anthers were protruding from the lower part of the ears and a second inoculation was made one week later after flowering. Ears inoculated and covered for 3 days showed infection

of the majority of spikelets and those covered for 7 days bore mycelium as a result of infection. Both *F. culmorum* and *F. avenaceum* became established with no difference in aggressiveness of the two species or in the nature and appearance of the lesions produced. Symptoms described were identical to those described by Atanasoff (1924) but *F. culmorum* produced coral coloured sporodochia between the glumes whereas those of *F. avenaceum* were apricot in colour.

Andersen (1948) studied infection of inoculated spring wheat ears by *Fusarium graminearum*, at five different stages of development. On ears at early anthesis, only those spikelets which had extruded anthers were infected. Those inoculated at booting did not become infected. Of those inoculated early flowering, 53 % of ears became infected whereas all the ears inoculated mid-flowering and afterwards became infected. This suggested that wheat ears differ in their susceptibility to *Fusarium* infection depending on the stage of ear maturity. Few or no grains were produced on those plants inoculated during flowering, however, grains from those inoculated at the end of anthesis and during grain development were progressively larger.

Takegami (1957 a and b) studied wheat spikelet morphology in relation to FEB and found that varieties which retained anthers inside the glumes tended to develop more severe blight infection in earlier stages of development. He speculated that the pathogen could attack more easily through the exposed part of the dead anther which was pushed behind the palea as the grain developed.

Explanations for the significance of anthesis in the development of FEB have centred on the isolation of stimulatory compounds from anthers. Pearce *et al.*(1976) ground and made extracts from anthers, paleas, lemmas, glumes, rachis, grain, leaves, stem and roots of wheat varieties

Marquis and Troll. A periodide assay was used to measure glycine betaine and choline from those extracts. Choline was measured to have 2201 units of stimulant activity when *F. graminearum* mycelial growth was measured by bioassay. Glycine betaine had 709 units of stimulant activity, seemingly being less potent as a stimulant. The periodide assay showed that considerably higher concentrations of choline and betaine were present in the anthers relative to the other plant organs. The authors suggested this as a possible reason for the susceptibility of plants during anthesis.

Strange and Smith (1978) examined the stimulatory properties of choline and betaine. They found that both promoted hyphal extension of *F. avenaceum*, *F. culmorum* and *F. graminearum* but not *M. nivale*. Neither compound had any effect on spore germination. They went further to state that neither choline nor betaine could be considered as specific primary determinants of susceptibility to *Fusarium* infection, since they also stimulated *Aspergillus niger in vitro*. Without pinpointing specific compounds, Strange and Smith proposed that wheatgerm extract contained other compounds stimulatory to *F. avenaceum* and *F. culmorum*. Obviously further investigations are needed to identify the stimulatory compounds of wheatgerm. Nkongolo *et al.* (1993) showed that betaine and choline caused an increase in mycelial growth of *F. graminearum* at concentrations ranging from $1\text{-}10^4$ µM, although there was no effect on spore germination. Anther extracts from the resistant cultivar Nobeoka Bozu stimulated mycelial growth of isolates but there was no stimulation of growth using anther extracts from the susceptible cultivar Laval-19. The authors suggested that factors other than the sensitivity of anthers seem to determine the relative susceptibilities of wheat ears at anthesis to *F. graminearum*.

Sturz and Johnston (1983) proposed that different species of *Fusarium* may infect ears at

different growth stages. At GS 45 (7 days prior to emergence from boot), they found that *Fusarium poae* and to a lesser extent, *F. avenaceum*, colonised ears and leaf sheaths prior to ear emergence from the boot. Thirty-two percent of the isolates on the lower ears of barley were *F. poae* and 37.5 % on the upper ear. Seven point nine percent of isolates on the lower ears of spring wheat were *F. poae* and 6.3 % on the upper ear. On the lower leaf sheaths this was 9.7 % and the upper leaf sheaths this was 10.4 %. Earlier colonisation of the ear and enclosing leaf sheath by species other than the predominant pathogenic species (*F. culmorum* and *F. graminearum*) may pre-dispose the tissue to infection by other *Fusarium* spp. later in the season. Sturz and Johnston, therefore, suggested that there is a successive pattern of infection by the different *Fusarium* species, beginning with *F. poae* and concluding with *F. culmorum* and *F. graminearum*.

Control of FEB

There are several methods to reduce the incidence and severity of FEB. Initially, the quantity and availability of inoculum may be reduced. Also, the infection of ears by the pathogen may be prevented by using resistant cultivars and applications of fungicides. Methods of cultural control to reduce the quantity of inoculum are discussed. The considerable research into the use of more resistant cultivars, biological control agents and fungicide applications which may reduce infection of ears, is also considered.

Cultural Control of FEB

Various cultural control measures may be implemented to reduce the risk of FEB epidemics occurring. Such cultural methods would help reduce the quantity of inoculum available for dispersal (Parry *et al.*, 1995).

When Martin and Johnston (1982) studied the control of *Fusarium* diseases of cereal grains in the Atlantic Provinces of Canada, they found that no single fungicide application or management practice offered total control of *Fusarium* diseases of cereals. However, according to Teich and Nelson (1984) substantial reductions in disease may be possible through practices of field sanitation, adequate fertilization and crop rotation. Also, it was shown that the wide host range of *Fusarium* species includes many weeds (Jenkinson and Parry, 1994a), and this may increase the difficulties of devising controls and the non-crop hosts in particular add a significant dimension to disease control.

Crop Rotations

Koehler (1924) observed a high incidence of FEB where wheat had been grown continuously or where it had been rotated with maize. In a survey of seven states of USA in 1919, Koehler found the incidence of ear blight was greater than 40 % if wheat was grown after maize, just under 30 % if it followed wheat and was decreased to less than 15 % if grown after Timothy and Clover. He related this increased incidence after maize to the presence of perithecia on cornstalks of susceptible cultivars and on the refuse of previous crops. The author suggested that cornstalks should be removed or ploughed in to bury refuse if wheat is to be sown afterwards and the sowing of wheat after maize should be avoided where possible. Comparable studies showed that the root rot of maize increased considerably where maize followed badly infected wheat in rotation. Koehler recommended a four year rotation for maize in the Corn Belt of the USA to hold ear blight in check.

When Teich and Nelson (1984) surveyed *Fusarium* ear blight in wheat fields in Canada, they also found that the previous crop was an important factor in the incidence of FEB. On average the incidence of FEB was six or seven times greater if wheat followed maize than if it followed

soybeans or other small grain cereals. The authors agreed with Koehler, in that, crop debris is the source of inoculum and that maize debris provided either more inoculum or the debris deteriorated less rapidly, hence crop rotations would help reduce the build-up of inoculum for FEB epidemics.

Land Preparation

The destruction of crop debris is a measure to reduce soil-borne inoculum. Cook (1980) isolated *Fusarium culmorum* and *F. graminearum* from humus in soil and Snyder and Nash (1968) found *F. culmorum* as chlamydospores in soil as a survival mechanism in the absence of host material. Crop debris (stubble) may act as a source of inoculum for epidemics. During surveys in Canada in 1983, Teich and Nelson (1984) found that if land was ploughed, there was a reduction in the number of ears with symptoms per 100,000 plants from 8.5 to 5.0, although this was shown not to be statistically significant.

As a result of field experiments using winter wheat inoculated with *F. graminearum* by Wilcoxson *et al.* (1992), the authors warned against the use of conservation tillage as this failed to bury thoroughly or destroy maize residues. Burning was suggested as the best method of destroying corn debris, however, since the European ban on stubble burning, mould board ploughing may be the next best method for reducing the available inoculum for the development of FEB epidemics. In a review of FEB, Parry *et al.*, (1995) also suggested that direct drilling or minimal tillage operations are likely to cause higher risks for FEB to develop.

Production Inputs

Martin *et al.* (1991) investigated the effect of production inputs on the incidence of infection by *Fusarium* species on cereal seed. Applications of nitrogen as ammonium nitrate were applied

at various growth stages to wheat, triticale and barley. The authors found that the high yielding production inputs including supplementary nitrogen top dressing and the plant growth regulator Ethephon (Cerone, 280g a.i ha^{-1}) favoured crop yield, but also favoured *Fusarium* infection of seed. For example, standard applications to barley of nitrogen plus ammonium nitrate plus a foliar fertiliser plus Ethephon caused an increase in the incidence of *F. avenaceum* to 22.2 % from 10.4 % for the standard application of 70 kg N per ha at seeding. In comparison, Teich and Nelson (1984) surveyed the incidence of FEB under different cultural practices and found that applications of urea caused a decrease in the incidence of FEB to 2.9 compared with 6.6 heads per 100,000 plants for ammonium nitrate treated plants. They concluded that the form of nitrogen in addition to the quantity applied affected the incidence of FEB. Teich and Nelson (1984) also recorded values for disease (recorded as the mean number of heads with symptoms per 100,000 ears) under high phosphorus rating of 4.4 and 7.9 for medium to low phosphorus applications, although the actual quantity applied was not presented. This suggested that low phosphorus inputs caused nutrient stress similar to that created by low nitrogen inputs, therefore increasing the susceptibility of plants to infection.

Weeds

Teich and Nelson (1984) showed that high weed densities significantly increased the number of ears with symptoms of FEB, although information on the densities of weeds were not presented. They also showed that where herbicides were used, the severity of FEB was reduced.

Jenkinson and Parry (1994a) collected weeds from 15 species and 10 families from fallow fields in Shropshire. The incidence of each *Fusarium* species isolated from stem bases was recorded. Over 70 isolates were obtained and tested for their pathogenicity to winter wheat cultivar

Mercia using spore suspensions. Several common broad-leaved weeds including *Capsella bursa-pastoris* (Shepherd's Purse), *Matricaria* spp. (Mayweed) and *Viola arvensis* (Field Pansy) were shown to provide alternative hosts for five species of *Fusarium* isolated and these isolates were shown to be pathogenic to winter wheat. Although the relative importance of weeds as sources of inoculum for the development of FEB of wheat was not determined by this study, the results demonstrate that weed species are a possible source of inoculum for the development of FEB epidemics. This suggests that weed control may be useful for the reduction of inoculum.

Biological Control

There is some evidence for the biological control of *Fusarium* seedling blight *in vitro* and in the glasshouse. For example, Tveit and Woods (1955), found that *Chaetomium cochliodes* and *C. globosum* demonstrated significant antagonistic properties against the *M. nivale* on oats. Millar and Colhoun (1969) demonstrated *Gliocladium roseum* decreased the incidence of disease when seed was inoculated with *M. nivale* but no control was shown for naturally infected seeds, suggesting that only surface infection of seeds was controlled by the antagonists. There has been little research into biological control of FEB, and little work on the impact of biocontrol of Fusarium seedling blight on subsequent ear blight development. Bateman (1979) investigated the relationship between *M. nivale* and the other micro-organisms on the seeds of wheat and barley. Wheat ears were inoculated at anthesis under glasshouse conditions with *M. nivale.* Grain contamination was subsequently reduced by inoculation of the wheat ears with the yeast *Sporobolomyces* spp. When ears were inoculated with *Cladosporium* spp, prior to anthesis the recovery of *M. nivale* from the seed was reduced from 36.5 % to zero. When wheat ears were inoculated before or after anthesis with *Alternaria* spp., seed infection, by *M. nivale*, was reduced from 36.5 % to 0 and 23.4 % respectively.

Wang *et al.*(1992) investigated the activity of *Bacillus subtilis* as a biological control agent, by spraying a cell suspension of the bacterium onto wheat plants at the same time as *Gibberella zeae* under glasshouse and field conditions. They found that *B. subtilis* showed strong inhibitory activity against the pathogen and reduced infection of spikelets of wheat by over 50 %.

Biological control of FEB and an understanding of the relationship between normal ear microflora and the FEB pathogens is necessary if more effective control of the disease is to be achieved, hence, this is an important area for future research (Flegg, 1993). In a review by Parry *et al.* (1995) it was suggested that the short time interval in which infection of the ears by FEB pathogens is achieved provides an ideal opportunity for biological control to be developed to reduce the disease and to minimise the risks associated with late applications of fungicides.

Genetic Resistance to FEB

There has been a considerable amount of screening work and breeding studies to identify sources of FEB resistance in wheat. Early work identified susceptible cultivars which were subsequently replaced by more resistant cultivars. Breeding studies have been carried out as a result of both naturally infected and artificially inoculated field trials. Genetic resistance of cereals to FEB has been reviewed extensively by Parry *et al.* (1995), Snijders (1990 and 1994) and Zhuping (1994).

Fungicide Control of Fusarium Ear Blight

Fungicide control of FEB is reviewed extensively in Chapter 3.

Aims of the Project

The objectives of the research described in this thesis were to:

(i) Assess the effects of fungicides on the incidence and severity of FEB in winter wheat.

(ii) Determine the most appropriate time of application of fungicides to control FEB.

(iii) Identify factors which affect fungicide efficacy against the pathogens.

There has been little research into fungicidal control of FEB despite numerous records which identified the disease as economically important worldwide. The primary objective of this work was to identify fungicides which were active against the pathogens. *In vitro*, glasshouse and field studies were undertaken to investigate fungicide efficacy and the most appropriate time of application to control the disease.

To understand the apparent inefficacy of fungicides reported in the literature, factors which may affect fungicidal control were also investigated. To this end investigations of the role of saprophytic microflora in disease control and a study of retention of fungicides on the ears of wheat to determine whether ear morphology and the stage of development of the ears influence fungicidal control of the disease were undertaken.

CHAPTER 2

General Materials and Methods

Pathogens

Table 1 lists the isolates used in the experimental work. All *Fusarium* and *Microdochium* species were obtained from John Innes Centre, Norwich, UK. Isolates of *Alternaria alternata* and *Cladosporium herbarum* were obtained from the International Mycological Institute, Egham, Surrey. The isolate of *Botrytis cinerea* was obtained from The University of Wolverhampton, UK.

Table 1 : Isolates used in the experimental work

Species	Isolate	Origin
Fusarium culmorum (W.G. Smith)	Fu42, Fu36, F200, Fu60, Fu5	UK
Sacc.	F302	France
Fusarium avenaceum (Fr.) Sacc.	F509, Fu11, Fu54	UK
	F303	France
Fusarium poae (Peck) Wollenw.	Fu53, F731, F733	UK
	F600	France
Fusarium graminearum Schwabe.	F507, F700, F604, F705	UK
Microdochium nivale var. *majus*	M71, M12, M15M2, -305/8	UK
Samuels and Hallett, formerly		
F.nivale (Fr.) Ces.		
Microdochium nivale var. *nivale*	RFN2I, -270/10, -213/21, M58	UK
Samuels and Hallett, formerly		
F.nivale (Fr.) Ces.		
Alternaria alternata (Fr.) Keissler	274016	UK
Botrytis cinerea (Pers.)	-	UK
Cladosporium herbarum (Pers.)	299105	UK
Link		

Culture and Storage of Pathogens

All aseptic operations were carried out in a sterile laminar flow cabinet. Glassware, media and sterile distilled water was autoclaved at 121°C and 103.4KPa for 20 minutes.

Isolates were sub-cultured onto potato dextrose agar (PDA, Oxoid, Unipath Ltd, Basingstoke, Hants) and sealed with Parafilm M (American Can Company, Greenwich, USA). After one to two weeks in an incubator at 20 °C +/- 2°C, cultures were transferred to the refrigerator for storage at 4°C. All isolates were sub-cultured after three months storage onto fresh media in order to maintain pathogenicity.

Spore Production

Sub-cultures of each isolate were produced by taking 5mm diameter plugs of inoculum from the edges of actively growing cultures using a sterile cork borer and transferring them onto plates of milled wheat agar (MWA, 15 g l^{-1} technical agar plus 5 g l^{-1} finely milled wheat grain). All sub cultures were then incubated in darkness at 20°C +/- 2°C for 7-14 days, after which time they were placed under near ultra violet light at 20°C +/- 2°C for 7-14 days to induce sporulation.

Preparation of Spore Suspensions for Experimental Use

Conidia and mycelium were washed from the surface of actively sporulating cultures using sterile distilled water. A sterile inoculating loop was used to dislodge the conidia and mycelium from the agar. Sterile distilled water (15ml) was used to rinse the conidia and mycelium through two layers of sterile muslin to remove the hyphal fragments. Spore concentrations were assessed using a haemocytometer (Weber Scientific International Limited, Middlesex, UK) and adjusted to the required concentration.

Culture of the Host (*Triticum aestivum* cultivar Avalon)

All seeds were treated with Beret (fenpiclonil) @ rate of 4 ml kg^{-1} seed, Novartis, Whittlesford, Cambridge, UK). For the glasshouse experiments, wheat seed was sown into trays containing a peat based compost (John Innes no.2) at a rate of approximately 200 seeds per tray. Seeds were allowed to germinate at 20 +/- 2°C on a glasshouse bench for seven days. When fully emerged, seedlings were vernalised for 8-10 weeks in an incubator set at 4 °C and with an eight hour photoperiod. After vernalisation, seedlings were transplanted to 15 cm diameter plastic pots containing John Innes no.2 compost, at a rate of five seedlings per pot. Plants were grown in a cool bay of the glasshouse (10+/- 3°C) and watered daily. The plants were treated for aphids using Aphox (0.9 g l^{-1} pirimicarb, Zeneca) and for mildew using Corbel (2.5 $g^{l}l$ fenpropimorph, BASF) as and when required.

Inoculation of Experimental Plants in the Glasshouse

Approximately 2 ml of conidial suspension (at a concentration of 200,000 conidia per ml of water) were applied to individual ears of winter wheat as a fine atomised spray, until run-off. All plants were mist irrigated for 6-12 hours following inoculation to provide continuous wetness conditions conducive to ear infection. Misting occurred every ten minutes for 15 seconds duration as determined by a leaf sensor set at ear height.

Fungicide Application

All fungicides were applied using a precision pot sprayer (custom built for Harper Adams Agricultural College by J. Reader), using Lurmark 110° flat fan nozzles (03-F110, Longstanton, Cambridge, UK), applying fungicides at 210 l ha^{-1}. In the field, applications were made using a pressurised knapsack sprayer (AZO carbon dioxide operated sprayer, AZO Sprayers, The Netherlands) with a four nozzle boom. Fungicides were applied at three bars pressure (1.2

l/min), in 180 l of water per ha using Lurmark 110° flat fan nozzles. The fungicides tested in glasshouse, field and *in vitro* experiments are listed in Table 2.

Table 2 : Fungicides used in field, glasshouse and *in vitro* studies

Active Ingredient		**Product Name**	**Field Rate**	**Manufacturer**
benomyl WP	500g l^{-1}	Benlate	0.5 l ha^{-1}	Du Pont, Stevenage, Herts
chlorothalonil	500g l^{-1} SC	Bravo 500	2 l ha^{-1}	BASF, Ipswich, Suffolk
fluquinconazole	250g l^{-1}WP		1 l ha^{-1}	AgrEvo, Hauxton, Cambridge
fluquinconazole	100g l^{-1} SC		1.5 l ha^{-1}	AgrEvo, Hauxton, Cambridge
flusilazole	400g l^{-1} EC	Genie	0.5 l ha^{-1}	Du Pont, Stevenage, Herts
flutriafol	125g l^{-1} SC	Pointer	1 l ha^{-1}	Zeneca, Haslemere, Surrey
prochloraz	450g l^{-1} EC	Sportak 45	0.9 l ha^{-1}	AgrEvo, Hauxton, Cambridge
pyrimethanil	4000g l^{-1} SC	Scala	3 l ha^{-1}	AgrEvo, Hauxton, Cambridge
tebuconazole	250g l^{-1} SC	Folicur	1 l ha^{-1}	Bayer plc, Basingstoke, Hants
copper-prochloraz	450 g l^{-1}EC	-	0.9 l ha^{-1}	AgrEvo, Hauxton, Cambridge

Disease Assessment

In field and glasshouse experiments, ears were inspected at Zadok's Growth Stage 75 (GS 75, Zadoks *et al.*, 1974) and assessed for ear blight symptoms. Severity of ear blight was expressed as the percentage of spikelets showing symptoms of infection.

Isolation and Identification of *Fusarium* spp From Harvested Grain

Grain was surface sterilised in 5% sodium hypochlorite solution (0.5 % available chlorine) for three minutes to eliminate surface contaminants. Grain was then rinsed in three changes of sterile distilled water and placed on sterile filter paper in a laminar flow cabinet to dry. Grain was placed into Petri-dishes containing approximately 15 ml of pda containing the antibiotics streptomycin sulphate (100 $\mu g\ ml^{-1}$), chloramphenicol (50 $\mu g\ ml^{-1}$) and neomycin sulphate (50 $\mu g\ ml^{-1}$) at a rate of five grains per plate. The plates were incubated at 20 °C +/-2°C for 7-14 days, after which time the isolates were identified. Isolates which could not be identified from potato dextrose agar (PDA) were sub-cultured onto sucrose nutrient agar to allow microscopic examination of spore morphology to aid identification. Refer to Appendix 1 for artificial media ingredients.

Statistical Analysis

All data were analysed using either analysis of variance or regression analysis procedures using Genstat 5 Version 3.1 (Lawes Agricultural Trust, Rothamsted Experimental station, Hertfordshire).

CHAPTER 3

The Effect of Eight Fungicides on Mycelial Growth, Conidial Germination and Germ Tube Extension of *Fusarium culmorum, F. avenaceum, F. poae, F. graminearum* and *Microdochium nivale*

Introduction

Control of FEB by fungicide sprays has proved to be poor and inconsistent. For example, Michail (1989) applied treatments including triadimefon (Bayleton Flowable 250g l^{-1}, Bayer plc), captafol plus triadimefon, (Bayleton DF, Bayer), fenpropimorph (Corbel 750g l^{-1}, BASF), carbendazim , (Derosal, 59.4%, Hoechst UK Ltd), propiconazole, (Desmel 250 gl^{-1}, Ciba-Geigy Agriculture), captafol plus pyrazophos (Furesan, 3.3 % / 16.6 % a.i., All India Medical) and prochloraz (Sportak 400g l^{-1}, AgrEvo UK Ltd, Hauxton, Cambridge, UK) to wheat at normal field rate at three different growth stages between GS 32 and GS 50. No treatment significantly reduced *Fusarium* infection of the ears.

One possible reason for such poor control is that ear blight is caused by a complex of species (Mesterhazy, 1984). For example, *F. graminearum, F. culmorum, F. avenaceum, F. poae* and *M. nivale* were amongst the 17 species associated with the disease in Hungary between 1970 and 1983. Hence, any activity against a sensitive species may be masked by subsequent infection by an insensitive species. Such observations have been made on the stem base of wheat plants where a number of species are present. Bateman (1993), for example, isolated stem-base pathogens from plots of wheat either untreated or treated with carbendazim (0.25 kg a.i ha^{-1}), prochloraz (0.4kg a.i ha^{-1}) or carbendazim plus prochloraz (as Sportak Alpha, 0.15kg a.i ha^{-1} + 0.40 kg a.i ha^{-1}). He found that following prochloraz application, the incidence of *Rhizoctonia cerealis* isolations increased whereas isolations of *Pseudocercosporella herpotrichoides* decreased.

There is little information available concerning the activity of products against individual *Fusarium* species in the field. However, some *in vitro* studies have focussed on the effects of individual active ingredients against single isolates of a species. For example, Richardson

(1970) showed that for a single isolate of *Fusarium graminearum*, 0.07-0.15 μg ml^{-1} of cyanomethyl mercury guanidine incorporated into PDA, reduced mycelial growth rate of the fungus by 50 %. These results should be considered with some reservation as only a single isolate of *F. graminearum* was studied.

In fungicide efficacy studies, it is important to note the adaptive capabilities of the causal organism used, illustrated by the development of resistance to methylbenzimidazole carbamate fungicides (MBCs) in *Microdochium nivale* (Locke *et al.*,1987). This was also reported by Pettitt *et al.* (1993) who isolated *Fusarium* species and *M. nivale* from stem base lesions of winter wheat grown throughout England and Wales. Over 90 % of the *M. nivale* isolates were resistant to 20μg ml^{-1} benomyl *in vitro*, however, none of the isolates of *F. avenaceum, F. culmorum* or *F. poae* showed resistance. Locke *et al.* (1987) suggested that there was little justification for the continuing use of MBC fungicides on wheat, except against FEB which may be caused by species other than *M. nivale.*

There is a limited amount of data on the efficacy of fungicides against FEB in the field. Jacobsen (1977) demonstrated the effect of several fungicides on Septoria leaf and glume blotch and *Fusarium* ear blight caused by *Fusarium graminearum* on grain yield and thousand grain weight of wheat. Benomyl (0.55 kg a.i ha^{-1}), mancozeb (1.76 kg a.i ha^{-1}), mancozeb plus benomyl (1.1 kg a.i ha^{-1} plus 0.27 kg a.i ha^{-1}) and benomyl plus carbendazim (0.55 kg a.i ha^{-1} plus 1.1kg a.i ha^{-1}) were applied at the GS 55 and 10 days later. Applications of benomyl alone or in combination with mancozeb or carbendazim gave 'cleaner' heads and less incidence of FEB. Fungicide applications resulted in increased thousand grain weights (up to 2 % increase over the control) for benomyl alone, 1.2 % for benomyl+mancozeb and 1.7 % for mancozeb alone and a reduction in the infected grain at harvest. It is unlikely that such small increases are

statistically different from the control. Ear blight was reduced from 24.3 % of grains infected for the control to 4 %. The lowest incidence was recorded for benomyl alone, however, all treatments reduced the percentage of ear blight by over 50 %. Although not confirmed experimentally, yield increases were attributed to increased grain numbers not grain weight itself. It was also suggested that the higher grain weights may have resulted from a longer grain filling period due to delayed senescence caused by application of fungicides. However, since this was not proved experimentally, further studies on fungicide effects on senescence are recommended. Although this shows apparently good control of FEB by fungicides, this was not borne out by other studies using different locations and with different disease pressures.

Studies in naturally-infected trials in the Atlantic Provinces of Canada by Martin and Johnston (1982) showed that plots which received propiconazole at a rate of 250g a.i ha^{-1} applied at GS 50 and GS 75 significantly reduced the incidence of FEB by 41 % compared to untreated control plots with a concurrent increase in yield of 34 %. Concentration of deoxynivalenol (DON), however, was not reduced significantly in the treated plots. It was recognised that no significant relationship existed between the severity of ear blight symptoms and DON concentration. They concluded that reductions or delays in symptom development, brought about by foliar fungicide applications, did not influence DON production. It must be remembered that applications of propiconazole would not only control *Fusarium* but also other foliar and ear diseases such as *Septoria nodorum* on the upper leaves (Jacobsen,1977). It is difficult therefore, to relate the 34 % yield increase directly to ear blight control.

Fehrmann and Ahrens (1984) artificially-inoculated wheat plants in the field with *Fusarium culmorum* at mid anthesis, and showed that applications of prochloraz (1.2 l ha^{-1}) before or after inoculation, resulted in yield losses of 34 % and 26 % respectively, compared to a yield

loss of 38 % for the control (untreated). For plants inoculated with *Fusarium graminearum*, a yield loss of 48 % in control plants was reduced to 36 % after a two applications of prochloraz. Unfortunately, the authors failed to indicate if such reductions were statistically significant.

Although guazatine (300g l^{-1}, Rappor, Dow Elanco) is formulated as a seed treatment for use on cereals and oilseed rape for the control of *Fusarium* spp, *Septoria nodorum* and *Tilletia caries*, Cameron *et al.* (1986) demonstrated that the fungicide gave good control of FEB in both naturally and artificially inoculated field trials when applied as a foliar spray. Two applications of 900 and 1200 g guazatine per hectare on rye at GS 55 resulted in significant yield increases to 45.9 and 46.2 dt ha^{-1} from 40.5 dt ha^{-1} for untreated plots. The incidence of infected ears was reduced from 19.7 % for untreated plots to 11.3 and 13.3 % for the two guazatine treatments, respectively. The yield increase, following guazatine application, was thus attributed to a decrease in the incidence of *Septoria* and *Fusarium* on the flag leaf and ear. It is unlikely that guazatine will be developed as a foliar spray in addition to its current use as a seed treatment due to development and registration costs together with the increased selection pressure for resistant strains of pathogens to guazatine.

Hutcheon and Jordan (1992) investigated fungicide timing and performance for *Fusarium* control in wheat. Glasshouse-grown wheat plants were inoculated at either flowering or at the seedling stage with *M. nivale*, *F. avenaceum*, *F. culmorum* or *F. graminearum*. UK264 (tebuconazole 250 l ha^{-1} EC and triadimenol 125 l ha^{-1}) effectively reduced the percentage of ear tissue infected by the four pathogens when applied 2,6 or 14 days after inoculation at anthesis. In untreated control plots 62.4 % of spikelets were infected which yielded 1.388 g of grain per ear. Plants inoculated at GS 49 and sprayed 3 days later were shown to bear ears

which had 33.6 % spikelets infected and which yielded of 1.543g grain per ear. Plants inoculated at GS 69 and sprayed three days later resulted in ears with 18.4 % of spikelets infected yielding 1.498g of grain per ear. In conclusion, the authors suggest that seed treatments can reduce disease in the early stages of plant development but are unlikely to prevent FEB later due to the importance of external inoculum. Also, late season foliar and ear sprays have the potential to reduce yield losses caused by severe ear blight disease. Hence, a combination of seed treatment and late season sprays were considered to be the two essential components for the control of FEB (Hutcheon and Jordan, 1992). Further work is required under field conditions to confirm the results of this work.

The relationship between FEB and mycotoxin concentration following fungicide application

Research by Boyacioglu *et al.*(1992) aimed to establish a relationship between disease severity, fungicide application and mycotoxin concentration. Using artificially-inoculated field trials of winter wheat, they found that the triazole fungicides triadimefon (Bayleton 60g a.i ha^{-1} Bayer) and propiconazole (Tilt 360g a.i ha^{-1} Ciba-Geigy) applied two days before inoculation, two days after inoculation and both two days before and two days after inoculation, reduced the number of grains infected by *F. graminearum* by 39-61 %. Also, the treatments significantly decreased the concentration of DON by 34.3-77.9 %. This compared to applications of thiabendazole (Mertect 340-F 120g a.i ha^{-1} MSD Agvet) applied at similar times and which had no apparent effect on disease incidence but caused a reduction in DON concentration by 83.4 % when applied two days pre-inoculation. Triadimefon and propiconazole reduced infection by *Fusarium graminearum* of wheat sprayed during and post inoculation. When applied during inoculation the DON level was reduced from 1.44 mg kg^{-1} for untreated plants to 0.37 and 0.63 mg kg^{-1} respectively. When applied post inoculation, DON was reduced from 1.37 to 0.35 and

0.36 mg kg^{-1}, respectively.

More recently, Milus and Parsons (1994), demonstrated the effects of a number of foliar fungicides against FEB of wheat under field conditions. These included benomyl (Benlate 50 DF 280g ai ha^{-1}), chlorothalonil (Bravo 750F 560g ai ha^{-1}), flusilazole (Punch 2E 140 g ai ha^{-1}), tebuconazole (Folicur 3.6 F 140g ai ha^{-1}), thiabendazole (Mertect LSP 2.9 F 280 g ai ha^{-1}), triadimefon and mancozeb (Bayleton 50 DF 70g ai ha^{-1} + Dithane 75DF 1.68 kg ai ha^{-1}). On evaluation, none of the foliar treatments significantly reduced ear blight incidence caused by *F. graminearum*, DON level or increased yield/test weight. It was suggested that although the fungicides had no control of FEB under severe disease pressure, it is possible that under naturally infected field conditions, they may be effective in controlling FEB.

It is evident that a complex relationship between fungicides and mycotoxin production exists. A reduction in the pathogen population may not always be associated with a reduction in mycotoxin concentration, as shown by Boyacioglu *et al* (1992). Further work using various pathogen populations should be carried out in order to study in more depth, the relationship between pathogen population, mycotoxin production and fungicide efficacy.

Seed Treatments and FEB control

One important aspect of FEB control is a reduction in seed infection which may result in seedling blight, crown and foot rots of a new crop. Historically, there has been much research into seed treatments for the control of *Fusarium* and *M. nivale* infection of seed and seedlings. However, the relationship between seed treatment and FEB control is poorly understood. Teich and Nelson (1984) reported that untreated seed did not necessarily produce greater symptoms of FEB at maturity and suggested that seed treatment would not inhibit infection of the

spikelets as late as anthesis. These observations were made on a comparison of seed treated with Vitaflo-280 and untreated seed. Subsequent studies by Teich and Hamilton (1985) contradicted this work. They found that seed treated with carboxin plus thiabendazole (Vitaflo-280, 360: 20 g l^{-1}), led to a decrease in the amount of FEB. No reasons to explain this contradiction were discussed. A larger study using a range of seed treatments would give a better picture of the connection between seed contamination with *Fusarium* and *Microdochium* species and the severity of FEB.

Although the relationship between Fusarium foot rot and ear blight has yet to be established, several workers showed that fungicides applied to reduce foot rot also reduced ear blight. For example, Liptoff and Lartaud (1983) demonstrated that a formulation of fenpropimorph and carbendazim (Corbel Duo, 373 g l^{-1} and 125 g l^{-1} respectively) gave effective control of Fusarium foot rot in wheat and barley. Also, a formulation of fenpropimorph and chlorothalonil (Corbel Star 200 g l^{-1} and 333 g l^{-1} respectively) applied as an ear wash caused a reduction in Fusarium ear blight. However, activity of fungicides against Fusarium foot rot, does not necessarily confer activity against FEB, and vice versa. Bateman *et al.*, (1981) showed that benomyl reduced the incidence of Fusarium foot rot. However Milus and Parsons (1994) showed that it failed to reduce ear blight caused by *F. graminearum* (Milus and Parsons, 1994).

Obviously, further studies of the epidemiology of the three Fusarium diseases of cereals; seedling blight, foot and crown rot and ear blight may contribute to an understanding of how seed treatments and the application of fungicides to control stem base Fusarium may contribute to the control of FEB.

In vitro screening of fungicide efficacy is commonly used to identify fungicides which show

activity against a particular plant pathogen. Rich *et al.* (1953) proposed that the use of *in vitro* screening methods, for example, the use of ED_{50} data and dose response curves, provided valuable information with which to select fungicides for field use. He did, however, identify factors which would affect the performance of fungicides under field conditions. These include the ability of the fungicide to withstand erosion by rainfall, hydrolysis, oxidation and its ability to adhere to the plant surface. Celetti and Hall (1987) investigated the effects of maneb, carbathiin and triadimenol when applied as seed treatments (1.95, 0.5 and 0.3 g a.i kg^{-1}, respectively) against *Fusarium* species *in vitro*. They found that carboxin was highly toxic against *F. avenaceum* between 1.1 and 5.3 mg l^{-1}. It reduced the diameter of mycelial growth of this species on fungicide-amended PDA by over 50 % at this concentration (ED_{50}). Similarly, the ED_{50} for maneb against a number of isolates of *F. culmorum* was 0.20-3.4 mg l^{-1} and for triadimenol against *F. equiseti* isolates was 0.9-1.5 mg l^{-1}. Further work in the field showed that maneb reduced the incidence of crowns infected by *F. avenaceum*, whereas the other two pathogens were not significantly affected. Carbathiin and triadimenol failed to cause any significant reduction in stem base symptoms caused by any species. Klein and Burgess (1987) also found that fenarimol and triadimefon significantly inhibited the mycelial growth of *F. graminearum* using Czapek Dox media amended with 100 µg ml^{-1} of the fungicides. Despite effects observed *in vitro*, both these fungicides failed to reduce white head formation and yield loss in field experiments. Obviously, this illustrates the disparity between *in vitro* and field performance of fungicides. This is discussed more fully in later chapters.

Fungicides used in the efficacy experiments

A number of fungicides were tested for their activity against five species of ear blight pathogen *in vitro.* (See Table 2 on page 36). Prochloraz is a non-systemic imidazole fungicide which has protectant and eradicant activity (Hassall, 1990), and which inhibits sterol biosynthesis.

Prochloraz has activity against some ascomycetes, deuteromycetes and some basidiomycetes, and is used against diseases such as leaf scald in barley (*Rhynchosporium secalis)*, eyespot in wheat *(Pseudocercosporella herpotrichoides)* and *Septoria* spp. in wheat (Hassall, 1990). Harris *et al.* (1979) showed that when prochloraz was applied at a rate of 400 g ai ha^{-1}, there was a significant reduction in the percentage of tillers infected with eyespot from 53.8 % for untreated plots to 31.2 % for plots treated with prochloraz. At this application rate, prochloraz also reduced Septoria severity to 7.3 % of leaf area affected from 21.2 % for untreated plots, and powdery mildew to 6.8 % of leaf area affected from 34.6 % for untreated.

Pyrimethanil is an anilino-pyrimidine fungicide. Evidence for the mode of action of pyrimethanil came from microscopy studies of *Botrytis fabae* on *Vicia faba* (Daniels and Lucas, 1995) and a study of extracellular proteins excreted by the pathogen during infection (Milling *et al.*, 1996). There was a significant reduction in germ tube extension and a significant decrease in the number of host cells killed at the individual penetration sites. There was an apparent reduction in the lytic activity of the pathogen towards the host epidermal cells. Subsequent analysis of extracellular proteins produced by *B. cinerea* in liquid culture showed that secretion of extracellular proteins (including hydrolases associated with pathogenesis) was inhibited. ^{14}C studies showed that inhibition of protein secretion of hydrolytic enzymes involved in pathogenesis may explain the mechanism of action of this fungicide (Milling *et al.* 1995). It was also shown that pyrimethanil inhibited methionine biosynthesis in *Botrytis cinerea* (Fritz *et al.*, 1997).

Chlorothalonil is a dithiocarbamate which is a foliar-applied fungicide with non-systemic activity. This fungicide is a tetra-chlorinated dinitrile of benzene commonly used in mixtures to widen the spectrum of activity and prevent the development of resistance as part of a

systemic / non-systemic pair. Chlorothalonil binds with thiol groups, since the fungitoxic activity of chlorothalonil is reversed in the presence of exogenous thiol groups (Vincent and Sisler, 1968).

Fluquinconazole, flutriafol, flusilazole and tebuconazole are all systemic 'azole' fungicides which inhibit ergosterol biosynthesis. Sterols (for example, ergosterol) are the major constituents of fungal membranes. They are constructed from acetyl units in such a way that the terminal C_{14} methyl from the acetyl group is removed and replaced by hydrogen atoms during the biosynthesis process. According to Hassall (1990), these fungicides act by preventing this demethylation process causing an accumulation of C_{14} methyl sterols, thereby preventing sterol manufacture. This then causes a disruption of membrane stability. According to Tomlin (1994), fluquinconazole shows protectant, eradicant and systemic activity and is active against some ascomycetes, deuteromycetes and basidiomycetes, for example, *Venturia inequalis* (the cause of apple scab) on apples and *Septoria* spp (the cause of leaf spot). and *Puccinia* spp. (rust) in wheat. Fluquinconazole was shown to reduce the mycelial growth of 44 monoconidial isolates of *V. inaequalis in vitro* (Schnabel and Parisi, 1997). The range of ED_{50} values was <0.01-1.60μg ai ml^{-1}. Flusilazole has systemic, protectant and curative activity and is active against *Venturia inequalis* and some major diseases damaging to cereals (Hassall, 1990). For example, applications of 400 g ai ha^{-1} of flusilazole were shown to reduce the percentage of stems infected with eyespot from 89.9 % in control plots to 64.1 % in treated plots, although the percentage of stems infected with sharp eyespot and brown foot rot was not significantly reduced (Bateman, 1990). According to Hassall (1990), flutriafol has contact and systemic activity with eradicant and protectant activity. By inhibiting ergosterol biosynthesis, it causes fungal cell wall collapse and inhibition of hyphal growth. It was suggested by Hassall (1990) that flutriafol shows activity against major leaf and ear diseases of cereals including

powdery mildew (*Erysiphe graminis),* leaf blotch (*Rhynchosporium secalis*), Septoria diseases *(Septoria* spp.), rusts *(Puccinia* spp.) and blight *(Helminthosporium* spp.) and the seed-borne diseases loose smut or bunt *(Tilletia caries) and* Fusarium diseases (*Microdochium nivale* and *Fusarium* species. Jenkyn *et al.*(1991), showed that flutriafol assessed at GS 30-31 or GS 32, significantly reduced the severity of take-all of winter barley when used as a seed treatment (Ferrax) but there was no significant increase in yield. At a rate of 400g ai ha $^{-1}$, flutriafol was also shown to significantly reduce the severity of *Rhizoctonia* root-rot of barley and cause an increase in grain yield (Cotterill, 1993). Tebuconazole (125 g ha^{-1}) has been shown to reduce *Septoria nodorum* on wheat (Loughman and Thomas, 1992) when applied at GS 61-65 and GS 73-83. Tebuconazole has also shown to be effective against *Rhizoctonia* root rot of barley (Cotterill, 1993), *Septoria tritici* of wheat (Milus, 1994) and yellow rust (*Puccinia striiformis*) of wheat (Jorgensen and Nielsen, 1994). Tebuconazole has also been shown to be effective against FEB. When applied either before or after inoculation, the percentage of spikelets infected with *Fusarium culmorum* and *F.graminearum* was significantly reduced to 16 % of spikelets infected compared with 41 % for untreated plants (Mauler-Machnik and Zahn, 1994).

Benomyl is a benzimidazole fungicide which acts by disrupting microtubule formation thereby inhibiting cell division (Davidse, 1986). It is used as a bulb / corm dip to control *Botrytis, Fusarium* spp. and *Sclerotinia* rots, a seed treatment for loose smut of winter wheat (*Ustilago nuga*) and *Ascochyta* of pea. It is also used as a soil treatment for *Verticillium* wilt of tobacco (Tomlin, 1994). There has been evidence of resistance to benomyl in many pathogens, including *M.nivale* (Locke *et al.*, 1987). The fungicide group, active ingredient and mode of action for the eight products investigated are summarised in Table 3.

The effect of these eight fungicides on mycelial growth, conidial germination and germ tube

elongation of *F. culmorum, F. avenaceum, F. poae, F. graminearum* and *M. nivale in vitro* was examined. In order to investigate the relationship between the ability of a selection of these fungicides to control pathogens *in vivo*, a series of field and glasshouse studies was undertaken to determine the relationship between fungicide, time of application and the incidence of disease, the severity of disease and the yield of winter wheat. All glasshouse and field studies undertaken are presented in Chapter 4.

Table 3. The fungicide group, active ingredient and mode of action of the eight products used during the experiments

Fungicide Group	Active Ingredient	Mode of Action
benzimidazole	benomyl	disrupts microtubule formation
dithiocarbamate	chlorothalonil	binds with thiol groups in dehydrogenase enzymes
triazole	fluquinconazole	inhibits ergosterol biosynthesis, by inhibiting the removal of 14α-methyl groups
triazole	flusilazole	as above
triazole	flutriafol	as above
triazole	tebuconazole	as above
imidazole	prochloraz	as above
anilino-pyrimidine	pyrimethanil	inhibition of protein secretion (hydrolytic enzymes)

Materials and Methods

The efficacy of eight fungicides on the mycelial growth of five *Fusarium* species was investigated by determining the diameter of mycelium of each pathogen on PDA amended with a range of concentrations of eight fungicides *in vitro* (Jenkinson, 1994). For each fungal isolate

(see Table 1, page 33), 5 mm plugs were placed centrally onto PDA plates amended with fungicides (see Table 2, page 36) at concentrations of 0.05, 0.25, 1.25 and 2.00 μg ai ml^{-1} of agar. For each fungicide and each concentration, five replicate plates were prepared. Plates with no fungicide provided control treatments. After inoculation, all plates were sealed with Parafilm and incubated in the dark for 5 days at 20°C +/- 2°C. After incubation, colony diameters recorded on fungicide amended media were compared with those recorded on control plates.

To determine the effect of fungicides on the germination of conidia, tap water agar (TWA) was amended with fungicides to produce concentrations of 0.05, 0.25 and 1.25 μg ai ml^{-1}. Unamended TWA was used as a control and each treatment had three replicates. Cellophane squares 1.5 cm diameter were boiled three times in distilled water to remove any inhibitory or stimulatory constituents and sterilised by autoclaving at 121°C and 103 Kpa for 15 minutes. Three cellophane squares were placed on the surface of each agar plate onto which aliquots of 25μl of spore suspension (100,000 conidia per ml) were dispersed. The isolates used in the spore germination experiment are listed in Table 4. Only isolates which sporulated readily *in vitro* were selected. Plates were incubated at 20°C +/- 2 °C for eight hours, after which time, cotton blue in lactophenol (15μl at 1 %) was added to each square. Nine hundred spores were assessed for germination (when the germ tube length exceeded the width of the spore) for each treatment. The germ tube lengths of 270 spores were measured for each treatment using an eyepiece graticule at a magnification of x40, the mean length calculated and measurement calibrated to μm using a microscope stage graticule.

Table 4. Isolates used in spore germination experiment

Fusarium and *Microdochium* species	Isolates
Fusarium culmorum	Fu42, F200
Fusarium avenaceum	Fu54, Fu11
Fusarium poae	Fu53
Microdochium nivale var. *majus*	M15M2
Microdochium nivale var. *nivale*	RFN2I, M58

Results

It was evident from the *in vitro* mycelial growth experiments that each of the five pathogens reacted differently to the fungicides tested. The effect of fungicide concentration on the diameter of mycelium growth of *F. culmorum, F. avenaceum, F. poae, M. nivale* and *F. graminearum* is shown in Table 5. Analysis of variance revealed no significant ($p<0.05$) difference between replicate plates of each treatment, however, there was a significant difference ($p<0.05$) between mycelial growth of the five species for the eight fungicides tested. There was greater variability between isolates of *M.nivale* and *F.graminearum* on both fungicide amended media and the control plates than for the other species. The analysis of variance showed that individual pathogen species responded differentially to the fungicides tested and to increasing concentration of fungicide.

For all species, prochloraz consistently gave the greatest reduction in the colony diameter. For example, the mean diameter of *F. culmorum* colonies was reduced from 69 mm for unamended PDA to 4 mm on PDA amended with 2 μg ai ml^{-1} and for *M. nivale* var. *majus* the colony diameter was reduced from 32 mm to 0 mm at these concentrations after 5 days. The mean colony diameter was reduced by increasing the fungicide concentration from 0 to 2 μg ml^{-1}. Tebuconazole and flusilazole also gave significant reductions of colony diameter of the five

pathogens. For example, the diameter of mycelium of *F. avenaceum* was reduced from 35 mm for control plates to approximately 6 mm and 8 mm at a concentration of 2μg ml^{-1} of tebuconazole and flusilazole respectively. Similarly, the mean colony diameter of *F. poae* was reduced from 60 mm to 5 mm and 8 mm for tebuconazole and flusilazole respectively at the highest concentration. Fluquinconazole and flutriafol failed to give effective reduction in mycelial growth of any species, even at the highest concentrations. Pyrimethanil failed to give effective reduction of mycelial growth of *Fusarium* species, however, it reduced the colony diameter of *M. nivale*. For *M. nivale* var. *majus*, the diameter of growth was reduced from 32 mm to approximately 7 mm after 5 days incubation and for *M.nivale* var. *nivale*, there was a reduction from 47 mm to 7 mm at 2μg ml^{-1} pyrimethanil. Also, benomyl caused effective reduction in mycelial growth of *F. culmorum*, *F. avenaceum*, *F. poae* and *F. graminearum* at 1.25 and 2 μg ml^{-1}, but the reduction in mycelial growth of *M. nivale* was variable. Approximately half of the isolates of *M. nivale* tested showed resistance to benomyl, however, the remainder showed a reduction in mycelial growth when inoculated onto PDA amended with benomyl.

The effect of fungicide concentration on the germination of conidia of selected isolates is shown in Table 6. Analysis of variance revealed a significant effect of fungicide and fungicide concentration ($p < 0.05$) on the percentage germination of conidia, however, there was no significant difference between replicates. Chlorothalonil consistently reduced the mean percentage of germinated conidia for all isolates, and, for the majority of isolates, germination was completely inhibited at 0.25 μg ml^{-1}. Most of the fungicides failed to cause a reduction in the germination of conidia at the concentrations tested after 8 hours incubation. Noticeable exceptions were that of benomyl which at concentrations of 1.25 μg ml^{-1}, and greater, caused a significant reduction in germination of both isolates of *M. nivale* var. *nivale* and

tebuconazole which reduced the germination of the same isolates to 14 and 13 % respectively at 2 μg ml^{-1}. It is possible that the var. *nivale* isolates were more sensitive to fungicides than the other species tested.

The germ tube length results (Table 7) showed that fungicides significantly reduced the germ tube length of conidia after 8 hours incubation. Chlorothalonil caused the most significant reduction of germ tube length of germinating conidia. For example, with *F. culmorum* (F200), average germ tube length was reduced from 3.80 to 0 μm at 0.25μg ml^{-1} chlorothalonil.

Table 5. The effect of concentration of eight fungicides on the diameter of *Fusarium culmorum, F. avenaceum, F. poae, Microdochium nivale* and *F. graminearum* colonies after 5 days incubation at 20°C +/- 2°C (number in parentheses are the standard errors)

		Mean Colony Diameter (mm)					
Fungicide	Concentration (μg ai ml^{-1})	*F.culmorum*	*F.avenaceum*	*F.poae*	*M.nivale var. majus*	*M.nivale* var. *nivale*	*F. graminearum*
tebuconazole	0.05	42.3 (9.74)	14.2 (2.35)	23.0 (4.33)	22.7 (18.51)	37.6 (16.03)	23.5 (3.55)
	0.25	17.2 (2.98)	10.6 (2.44)	18.7 (4.35)	19.4 (15.90)	29.7 (12.63)	18.4 (4.16)
	1.25	8.2 (3.86)	7.4 (2.88)	15.7 (3.85)	6.7 (5.88)	20.0 (8.97)	10.9 (3.66)
	2.00	5.0 (3.88)	5.8 (3.18)	11.0 (5.31)	3.9 (4.78)	18.7 (9.23)	9.7 (3.80)
flusilazole	0.05	48.6 (5.27)	19.2 (3.70)	39.2 (7.60)	19.2 (13.89)	30.2 (10.54)	30.6 (5.84)
	0.25	19.7 (6.47)	16.5 (3.24)	25.9 (5.62)	13.1 (8.89)	24.2 (6.86)	19.9 (5.64)
	1.25	9.5 (5.99)	11.1 (3.15)	17.7 (6.88)	2.9 (1.76)	14.8 (6.29)	11.7 (4.20)
	2.00	7.0 (4.48)	8.4 (1.26)	15.4 (7.61)	1.6 (1.14)	10.1 (3.70)	8.4 (2.76)
prochloraz	0.05	16.0 (3.52)	8.9 (1.32)	23.8 (5.46)	5.3 (4.58)	16.5 (8.46)	15.1 (4.24)
	0.25	8.9 (3.93)	4.5 (3.12)	11.9 (6.64)	1.3 (1.25)	9.1 (3.81)	10.1 (3.72)
	1.25	6.2 (4.11)	1.4 (1.93)	6.6 (5.92)	0.1 (0.22)	4.1 (3.52)	3.8 (2.21)
	2.00	4.4 (3.22)	0.9 (1.23)	2.5 (3.05)	0 (0)	2.1 (2.13)	2.2 (1.72)

Note. Mean of 4 isolates for each species

Table 5. Contd

Fungicide	Concentration (μg ai ml^{-1})	Mean Colony Diameter (mm)					
		F.culmorum	*F.avenaceum*	*F.poae*	*M.nivale var. majus*	*M.nivale* var. *nivale*	*F. graminearum*
fluquinconazole	0.05	66.55 (9.68)	35.55 (9.21)	60.65 (9.92)	28.45 (19.71)	50.00 (10.72)	41.30 (12.11)
	0.25	63.10 (5.46)	30.70 (7.52)	60.80 (7.73)	27.20 (19.18)	48.95 (8.79)	51.70 (8.90)
	1.25	56.35 (4.00)	24.05 (5.09)	42.95 (6.13)	26.45 (18.93)	36.20 (5.64)	49.40 (3.47)
	2.00	52.20 (8.28)	20.15 (4.49)	36.50 (3.89)	22.60 (16.36)	31.95 (10.81)	48.60 (3.83)
pyrimethanil	0.05	57.85 (4.08)	26.10 (6.06)	48.15 (3.33)	18.80 (17.05)	34.95 (12.01)	49.30 (15.31)
	0.25	59.35 (5.20)	28.85 (5.43)	45.65 (3.08)	12.15 (12.50)	17.90 (6.82)	56.20 (10.34)
	1.25	56.60 (4.02)	30.15 (4.21)	44.95 (2.39)	9.05 (12.91)	10.15 (5.74)	54.80 (6.28)
	2.00	54.55 (4.01)	28.70 (4.60)	39.50 (4.31)	6.65 (11.11)	7.20 (7.58)	49.65 (8.28)
chlorothalonil	0.05	61.65 (5.08)	28.45 (8.09)	55.65 (2.94)	25.65 (18.91)	23.50 (9.55)	37.30 (12.19)
	0.25	40.55 (6.82)	24.65 (8.57)	43.15 (5.92)	23.80 (17.26)	19.90 (10.36)	31.95 (7.96)
	1.25	26.00 (6.13)	21.30 (5.29)	33.05 (7.61)	15.80 (13.70)	14.80 (10.47)	19.90 (7.59)
	2.00	26.75 (7.68)	20.95 (5.79)	27.30 (7.98)	11.25 (12.69)	13.80 (11.14)	17.30 (7.95)

Table 5. Contd

Fungicide	Concentration (μg ai ml^{-1})	Mean Colony Diameter (mm)					
		F.culmorum	*F.avenaceum*	*F.poae*	*M.nivale var. majus*	*M.nivale* var. *nivale*	*F. graminearum*
flutriafol	0.05	80.00 (0)	37.90 (11.72)	74.10 (7.09)	34.75 (25.79)	41.40 (14.07)	72.85 (8.54)
	0.25	74.95 (5.30)	32.90 (10.76)	59.00 (7.76)	32.70 (23.48)	35.50 (12.22)	70.45 (9.23)
	1.25	60.40 (9.71)	24.20 (5.12)	47.85 (4.78)	29.95 (17.76)	25.45 (6.64)	65.65 (17.85)
	2.00	49.7(14.01)	21.50 (3.68)	43.55 (5.82)	28.45 (17.71)	21.90 (6.25)	64.10 (12.78)
benomyl	0.05	67.30 (5.97)	32.90 (12.09)	60.50 (9.63)	24.05 (17.26)	31.35 (15.21)	40.80 (8.99)
	0.25	64.05 (6.23)	31.40 (11.73)	52.75 (6.65)	11.55 (18.08)	25.05 (20.28)	47.80 (11.05)
	1.25	32.40 (2.30)	24.85 (10.34)	25.15 (4.75)	8.20 (11.74)	23.60 (18.57)	18.95 (2.91)
	2.00	10.25 (1.86)	22.80 (8.17)	5.60 (4.61)	7.30 (10.42)	21.95 (18.11)	11.20 (3.07)
control	-	68.98 (5.27)	35.38 (7.44)	59.93 (2.53)	32.34 (25.97)	47.19 (21.55)	50.58 (7.24)

SED (fungicide) = 0.583, 7df
SED (conc) = 0.461, 4df
SED (species) = 0.505, 5df
SED (fungicide*concentration) = 1.305, 28df
SED (fungicide* species) = 1.429, 35df
SED (concentration* species) = 1.130, 20df
SED (fungicide*concentration*isolate) = 3.196, 140df

Table 6. The effect of concentration of eight fungicides on the percentage of conidia germinated for *F.culmorum, F.avenaceum, F.poae* and *Microdochium nivale* after 8 hours following inoculation onto fungicide amended PDA (number in parentheses is the standard error)

		Mean Percentage of Spores Germinated							
Fungicide	Conc µg.ai. ml^{-1}	*F. culmorum* Fu42	*F. culmorum* F200	*F.avenaceum* Fu54	*F.avenaceum* Fu11	*F. poae* Fu53	*M. nivale* var. *majus* M15M2	*M. nivale* var. *nivale* RFN2I	*M. nivale* var. *nivale* M58
tebuconazole	0	97.67(0.71)	99.22(0.83)	96.00(1.22)	96.56(1.01)	82.67(1.58)	97.00(0.71)	91.33(3.35)	77.00(4.12)
	0.05	97.22(0.84)	98.33(1.00)	94.00(1.22)	95.44(1.01)	82.67(2.69)	94.78(0.94)	83.89(2.09)	78.11(4.34)
	0.25	97.11(0.78)	98.56(0.88)	89.67(3.24)	94.43(1.12)	84.11(2.93)	86.00(3.04)	73.22(3.49)	74.67(3.00)
	1.25	97.22(0.67)	98.67(0.87)	81.33(2.60)	94.00(1.66)	83.22(1.64)	80.22(3.22)	14.22(1.92)	13.56(2.55)
flusilazole	0	98.56(1.01)	98.22(0.97)	94.78(1.20)	98.56(1.01)	81.00(1.41)	95.67(0.97)	92.78(0.97)	91.67(1.32)
	0.05	98.11(1.27)	98.00(1.41)	89.44(1.42)	97.78(1.30)	79.67(1.32)	95.00(1.12)	91.11(0.78)	89.78(0.67)
	0.25	98.00(0.87)	96.33(2.55)	82.89(6.60)	97.22(1.09)	80.44(2.13)	94.56(1.58)	91.56(1.60)	80.11(3.89)
	1.25	97.22(1.20)	96.89(1.36)	79.22(4.68)	97.78(0.67)	79.22(1.20)	93.33(1.22)	82.67(2.87)	72.00(2.29)
prochloraz	0	97.78(0.83)	99.11(0.78)	97.89(1.27)	97.33(0.71)	82.67(2.29)	97.78(1.09)	81.33(3.28)	21.44(2.55)
	0.05	97.67(0.71)	98.44(1.01)	92.89(2.76)	96.78(1.09)	85.33(2.12)	94.22(0.83)	83.00(3.00)	20.56(3.05)
	0.25	97.67(0.87)	98.11(1.05)	86.00(1.32)	97.00(0.71)	84.00(2.69)	97.00(0.71)	80.33(2.60)	20.56(2.07)
	1.25	96.89(2.37)	98.44(1.13)	84.33(3.28)	95.33(1.22)	85.22(2.82)	96.44(1.01)	79.78(2.73)	18.44(2.19)

Table 6. Contd

Fungicide	Conc µg.ai. ml^{-1}	Mean Percentage of Spores Germinated							
		F. culmorum Fu42	*F. culmorum* F200	*F.avenaceum* Fu54	*F.avenaceum* Fu11	*F. poae* Fu53	*M. nivale* var. *majus* M15M2	*M. nivale* var. *nivale* RFN2I	*M. nivale* var. *nivale* M58
fluquinconazole	0	95.00(2.06)	94.78(1.92)	96.56(1.86)	96.33(1.22)	13.33(2.64)	91.11(5.51)	23.00(3.24)	21.56(5.02)
	0.05	95.00(2.00)	97.56(2.19)	94.78(3.20)	95.44(1.01)	13.22(1.72)	89.78(4.27)	22.89(1.83)	21.56(2.01)
	0.25	93.67(2.18)	92.00(2.55)	95.78(1.45)	93.89(1.62)	14.11(2.57)	85.56(4.03)	21.44(2.92)	19.78(1.39)
	1.25	93.22(2.11)	95.22(1.72)	91.78(1.73)	93.11(1.36)	13.33(2.65)	82.44(4.61)	19.11(5.16)	17.78(3.70)
pyrimethanil	0	97.11(1.05)	97.78(0.83)	84.67(1.99)	96.33(1.58)	11.89(2.32)	95.22(0.83)	23.44(2.65)	22.22(2.05)
	0.05	96.33(1.00)	97.78(0.97)	75.33(4.02)	94.56(1.33)	11.67(2.29)	92.67(1.00)	22.33(1.87)	20.89(2.03)
	0.25	96.44(0.88)	97.33(1.00)	71.89(5.24)	93.67(1.12)	12.56(2.51)	89.44(1.81)	20.33(2.24)	17.78(1.56)
	1.25	96.33(1.22)	97.00(1.00)	73.89(4.39)	93.00(2.12)	12.11(3.10)	82.22(5.17)	17.22(1.86)	15.67(2.87)
chlorothalonil	0	98.11(1.05)	97.44(0.88)	79.67(3.46)	97.78(0.97)	15.33(1.32)	95.11(0.78)	28.78(3.46)	27.33(3.67)
	0.05	81.11(3.76)	8.78(1.56)	1.22(1.42)	8.89(2.20)	0.00(0)	0.00(0)	0.00(0)	0.00(0)
	0.25	0.00(0)	0.11(0.33)	0.00(0)	1.67(1.13)	0.00(0)	0.00(0)	0.00(0)	0.00(0)
	1.25	0.00(0)	0.00(0)	0.00(0)	0.00(0)	0.00(0)	0.00(0)	0.00(0)	0.00(0)

Table 6. Contd

Fungicide	Conc µg.ai. ml^{-1}	Mean Percentage of Spores Germinated							
		F. culmorum Fu42	*F. culmorum* F200	*F.avenaceum* Fu54	*F.avenaceum* Fu11	*F. poae* Fu53	*M. nivale* var. *majus* M15M2	*M. nivale* var. *nivale* RFN2I	*M. nivale* var. *nivale* M58
flutriafol	0	99.00(1.12)	96.44(1.33)	94.44(1.51)	96.33(1.73)	10.78(0.97)	97.44(1.01)	26.44(2.47)	26.00(2.06)
	0.05	98.22(1.20)	95.78(1.09)	93.78(1.48)	96.33(1.50)	10.56(1.42)	95.78(0.97)	22.11(2.47)	24.44(2.74)
	0.25	98.22(1.30)	94.67(1.00)	92.89(2.26)	96.11(1.27)	10.22(1.48)	95.33(1.00)	20.67(1.22)	24.22(1.79)
	1.25	98.11(1.36)	94.67(1.04)	92.56(1.94)	93.56(1.42)	9.78(1.49)	94.44(1.13)	18.67(1.41)	22.33(1.73)
benomyl	0	98.78(0.83)	98.33(1.22)	85.33(3.53)	96.22(0.97)	79.89(2.52)	94.33(0.87)	79.89(2.76)	20.11(2.62)
	0.05	98.44(0.88)	97.67(1.00)	84.11(1.62)	96.44(1.01)	78.11(1.62)	94.78(1.09)	78.33(1.00)	15.89(1.83)
	0.25	97.78(0.83)	97.56(0.73)	83.22(1.39)	96.67(0.87)	74.78(3.27)	93.89(1.27)	78.33(1.22)	7.44(1.59)
	1.25	97.67(1.00)	97.33(0.87)	82.22(2.17)	95.89(1.27)	86.22(2.54)	75.22(2.86)	9.44(4.13)	1.00(1.00)

SED (fungicide) = 0.236, 7df
SED (concentration) = 0.1672, 3df
SED (isolate) = 0.2364, 7df
SED (fungicide*concentration) = 0.4729, 21df
SED (fungicide*isolate) = 0.6688, 49 df

Table 7. The effect of fungicide concentration on the mean germ tube length (μm) of conidia after 8 hours following inoculation onto fungicide amended PDA for *F.culmorum, F.avenaceum, F.poae* and *Microdochium nivale* (number in parentheses is the standard error)

		Mean Germ Tube Length (μm)							
Fungicide	Conc	*F.culmorum*	*F. culmorum*	*F.avenaceum*	*F.avenaceum*	*F. poae*	*M. nivale* var. *majus*	*M. nivale* var. *nivale*	*M. nivale* var. *nivale*
		Fu42	F200	Fu54	Fu11	Fu53	M15M2	RFN2I	M58
tebuconazole	0	4.15(0.33)	14.49(1.23)	8.04(0.67)	13.34(1.30)	8.30(0.75)	4.24(0.37)	5.3(0.42)	9.45(0.90)
	0.05	4.24(0.33)	12.63(0.83)	6.10(0.51)	12.37(1.19)	8.22(0.69)	3.27(0.21)	3.89(0.30)	10.42(0.95)
	0.25	3.98(0.33)	12.46(1.12)	5.57(0.46)	12.63(1.82)	7.69(0.68)	3.09(0.22)	2.74(0.09)	9.10(0.74)
	1.25	3.00(0.17)	12.46(0.96)	5.3(0.44)	9.89(0.99)	5.39(0.48)	2.74(0.09)	2.65(0.18)	8.48(0.79)
flusilazole	0	4.77(0.43)	10.25(0.67)	5.83(0.53)	11.22(0.92)	9.63(0.68)	3.29(0.21)	6.27(0.47)	9.63(0.91)
	0.05	4.06(0.30)	7.69(0.65)	6.54(0.52)	10.51(0.93)	7.33(0.63)	3.18(0.20)	4.68(0.37)	8.48(0.85)
	0.25	3.80(0.30)	8.92(0.77)	6.10(0.51)	9.81(0.74)	8.22(0.72)	3.00(0.17)	4.59(0.33)	7.89(0.70)
	1.25	4.15(0.33)	9.54(0.71)	5.65(0.44)	8.30(0.70)	6.10(0.46)	2.92(0.15)	2.83(0.12)	8.39(0.70)
prochloraz	0	4.82(0.34)	10.69(0.62)	8.22(0.68)	17.31(1.59)	9.01(0.70)	3.18(0.23)	4.59(0.36)	8.48(0.73)
	0.05	4.68(0.35)	10.16(0.62)	8.13(0.70)	13.60(1.45)	8.75(0.67)	2.65(0.18)	3.00(0.17)	7.51(0.62)
	0.25	4.06(0.30)	8.13(0.65)	7.51(0.58)	12.54(1.18)	7.86(0.69)	2.83(0.13)	3.09(0.22)	7.07(0.64)
	1.25	3.36(0.25)	9.19(0.67)	7.95(0.58)	11.40(0.98)	7.60(0.66)	2.65(0.18)	2.74(0.09)	7.51(0.61)

Table 7. Contd

Fungicide	Conc	Mean Germ Tube Length (μm)							
		F. culmorum Fu42	*F. culmorum* F200	*F.avenaceum* Fu54	*F.avenaceum* Fu11	*F. poae* Fu53	*M. nivale* var. *majus* M15M2	*M. nivale* var. *nivale* RFN2I	*M. nivale* var. *nivale* M58
fluquinconazole	0	7.59(0.49)	6.27(0.43)	7.69(0.63)	9.36(0.75)	10.69(0.94)	2.92(0.15)	3.89(0.33)	10.07(0.65)
	0.05	6.89(0.60)	6.27(0.50)	8.39(0.71)	9.28(0.63)	7.86(0.65)	2.65(0.18)	3.18(0.20)	8.48(0.51)
	0.25	7.16(0.48)	6.01(0.44)	7.07(0.53)	9.01(0.73)	8.57(0.64)	2.74(0.09)	3.18(0.20)	8.39(0.49)
	1.25	6.97(0.48)	5.57(0.41)	6.71(0.47)	8.48(0.56)	7.42(0.63)	2.83(0.12)	3.45(0.26)	7.42(0.50)
pyrimethanil	0	5.83(0.45)	6.01(0.47)	8.13(0.57)	10.25(1.01)	7.60(0.38)	3.09(0.18)	3.27(0.24)	8.13(0.54)
	0.05	5.74(0.53)	5.92(0.43)	6.18(0.51)	8.04(0.59)	6.71(0.44)	3.09(0.18)	3.36(0.25)	8.13(0.54)
	0.25	5.57(0.43)	6.54(0.40)	5.57(0.43)	8.48(0.70)	4.77(0.37)	3.36(0.25)	3.80(0.30)	8.92(0.53)
	1.25	6.18(0.53)	6.80(0.43)	5.12(0.42)	7.95(0.67)	5.48(0.49)	2.83(0.12)	3.00(0.17)	8.13(0.54)
chlorothalonil	0	3.80(0.30)	5.83(0.48)	5.21(0.30)	7.33(0.63)	5.48(0.49)	4.24(0.37)	4.59(0.36)	8.75(0.53)
	0.05	0(0)	2.83(0.12)	0(0)	2.21(0.38)	0(0)	0(0)	0(0)	4.68(0.35)
	0.25	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	1.33(0.25)
	1.25	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)

Table 7. Contd

		Mean Germ Tube Length (μm)							
Fungicide	Conc	*F. culmorum*	*F. culmorum*	*F.avenaceum*	*F.avenaceum*	*F. poae*	*M. nivale* var. *majus*	*M. nivale* var. *nivale*	*M. nivale* var. *nivale*
		Fu42	F200	Fu54	Fu11	Fu53	M15M2	RFN2I	M58
flutriafol	0	2.65(0.18)	9.72(0.50)	6.89(0.47)	15.72(1.07)	8.57(0.68)	3.53(0.26)	3.18(0.23)	8.3(0.54)
	0.05	2.65(0.18)	9.19(0.47)	7.16(0.49)	12.81(0.97)	8.57(0.58)	3.27(0.24)	2.92(0.15)	8.3(0.56)
	0.25	2.65(0.18)	8.66(0.44)	7.16(0.49)	13.69(0.82)	7.60(0.52)	2.83(0.12)	2.74(0.09)	6.98(0.41)
	1.25	2.65(0.18)	8.39(0.40)	6.89(0.49)	13.25(1.04)	7.24(0.54)	3.09(0.18)	2.92(0.15)	7.60(0.58)
benomyl	0	4.06(0.35)	9.98(0.76)	7.42(0.58)	18.02(1.52)	8.48(0.64)	3.09(0.22)	4.68(0.42)	9.89(0.85)
	0.05	4.42(0.34)	10.07(0.63)	8.13(0.62)	17.93(1.75)	9.01(0.66)	2.92(0.15)	4.59(0.36)	9.19(0.64)
	0.25	3.62(0.30)	9.98(0.79)	7.60(0.50)	15.19(1.50)	8.04(0.60)	2.65(0.18)	3.62(0.24)	8.22(0.71)
	1.25	3.27(0.24)	9.81(0.58)	6.10(0.48)	14.66(1.52)	5.12(0.42)	0.80(0.23)	2.65(0.18)	8.75(0.57)

SED (fungicide) = 0.1393, 7df
SED (concentration) = 0.0985, 3df
SED (isolate) = 0.1477, 7df
SED (fungicide*concentration) = 0.2785, 21df
SED (fungicide*isolate) = 0.4178, 49 df
SED (concentration*isolate) = 0.2954, 21 df
SED (fungicide*concentration*isolate) = 0.8355, 147 df

Discussion

The imidazole fungicide prochloraz was consistently the most effective fungicide at reducing the mycelial growth of all five pathogenic species *in vitro*. Over 90 % reduction in the diameter of mycelium was observed for the 24 isolates tested. Polley *et al.* (1991) also observed effective reduction of growth of ear blight pathogens by this fungicide when tested using fungicide-amended media. He observed complete inhibition of mycelial growth of over 80 % of the *M. nivale* isolates at concentrations of 0.05µg ml^{-1} of agar. This compares favourably with the results for this study.

The reduction in mycelial growth by tebuconazole, flusilazole, prochloraz and chlorothalonil compares with data for the ED_{50} for maneb, carbathiin and triadimenol when tested against *F. avenaceum* which occurred in the range of 1.1-5.3 mg l^{-1}. The ED_{50} for *F. culmorum* varied between 0.2 and 3.4 mg l^{-1} for these fungicides (Celetti and Hall, 1987). This is interesting in that triadimenol, like tebuconazole, flutriafol, fluquinconazole and flusilazole, is a demethylation inhibitor from the triazole group (Hassall, 1990), hence, the mode of action is the same. In a report by Klein and Burgess (1987), the pyrimidine fungicide fenarimol (also a DMI) and the triazole fungicide triadimefon showed effective reduction of mycelial growth of *F. graminearum in vitro* at concentrations of 100 µg ml^{-1}. However, this concentration is considerably higher than the concentrations used in the experiments presented here at which similar levels of growth reduction were observed. Also, these results show that the triazole fungicides flutriafol and fluquinconazole failed to give such effective control of the five species tested, as observed for maneb, carboxin and triadimenol by Celetti and Hall (1987). Hence there appears to be differences in the activity of different fungicides against these pathogens within the same chemical group, due to inherent differences in the site of activity and uptake of these fungicides.

In contrast to the triazole fungicides, chlorothalonil showed greater activity against spore germination of the pathogens than mycelial growth. Chlorothalonil consistently inhibited the germination of conidia after 8 hours incubation, illustrating its broad spectrum of activity. According to Gasztonyi and Lyr (1995), chlorothalonil acts to inhibit normal cell metabolism by reacting with thiol groups in cells, such as contained within glutathione The results for benomyl were interesting because this fungicide gave effective reduction of the mycelial growth of *Fusarium* species but not *Microdochium nivale*. Certain isolates showed no reduction in the diameter of mycelial colonies with increasing concentration of benomyl. These results indicate the possible development of resistance to benzimidazole fungicides which has been reported in many plant pathogens including *M. nivale*. For example, resistance to benomyl has been reported in *Neurospora crassa* (Borck and Brayma, 1974), *Botrytis cinerea* (Yarden and Katan, 1993), *Rhynchosporium secalis* (Wheeler *et al.*, 1994) and *Aspergillus nidulans* (van Tuyl, 1977). Locke *et al.*(1987) reported on the widespread resistance to MBC fungicides in populations of *M. nivale.* He sampled stem bases in England and Wales in 1986 and found that 92.1 % of *M. nivale* isolates were resistant to benomyl. He therefore recommended that the use of this fungicide as a seed treatment for the control of *M. nivale* on wheat could not be justified.

Pyrimethanil has been shown to reduce the diameter of mycelium of *Venturia inaequalis* by 50 % at concentrations of between 0.23 and 33.8 μg ai ml^{-1} agar (Schnabel and Parisi, 1997). Due to the novel mode of action of pyrimethanil, PDA was an inappropriate medium for testing the efficacy of this fungicide *in vitro*. Milling *et al.* (1995) reported that pyrimethanil interferes with the production of complex proteins such as extracellular proteins and hydrolases involved in pathogenicity. In PDA, amino acids are available for the fungus to use in the production of extracellular proteins, hence, the interference of pyrimethanil with the production of

extracellular proteins within fungal hyphae is prevented. Masner *et al.* (1994) showed that pyrimethanil inhibited the mycelial growth of *Botrytis cinerea, Pseudocercosporella herpotrichoides* and *Helminthosporium oryzae* on defined media lacking amino acids. For this reason, the use of more defined and less nutrient rich media would provide a more useful determination of the efficacy of pyrimethanil against *Fusarium* species *in vitro*. This does not however, explain the sensitivity of *Microdochium nivale* to this fungicide *in vitro*. There was a significant reduction in the diameter of mycelial growth of *M. nivale* when inoculated onto PDA amended with pyrimethanil. Obviously, this is a different species and the mechanism of utilisation of available nutrients for the manufacture of extracellular proteins may be different for this species. This may be an interesting area for further research.

Despite the inherent problems encountered whilst interpreting the results from *in vitro* studies alone, such studies provide valuable means of determining fungicide efficacy against individual species of pathogen, rather than the disease complex itself (Rich *et al.*, 1953). Problems in interpretation arise because of difficulty in comparing the germination values for different species on fungicide amended media. This is because the initial rate of conidium germination may be an important factor affecting the rate of inhibition of fungal germination and mycelial growth caused by these fungicides. For example, Pontzen and Scheinpflug, (1989) found that the inhibition of *Venturia inaequalis, Botrytis cinerea* and *Puccinia graminis* by sterol inhibiting fungicides such as bitertanol, triadimenol and tebuconazole, depended upon the sterol reserves of the conidium. *Botrytis cinerea* and *V. inequalis* have no sterol reserve, hence biosynthesis occurs within 1-2 hours of germination, therefore, these species are inhibited by fungicides more quickly. *Puccinia graminis* conidia contain large sterol reserves, therefore, sterol biosynthesis begins 6-8 hours after germination and this species is less affected by fungicides. Lees (1995) found that the rapid germination of *Microdochium nivale* compared

with *F. culmorum* and *F. avenaceum* resulted in increased incidence of inhibition of conidial germination of this species by flusilazole and prochloraz. Obviously, the study of sterol biosynthesis in *Fusarium* species and *Microdochium nivale* would clarify this and is an area for further work.

The *in vitro* experiments provided useful information for the selection of fungicides for efficacy and experiments *in vivo* using glasshouse and field experiments. The results showed the efficacy of fungicides against individual species which is particularly useful because under field conditions 17 species have been associated with the disease (Mesterhazy, 1984). On the basis of the mycelial growth tests, prochloraz and tebuconazole were selected for further trials in the glasshouse and field, since these two products effectively reduced the diameter of mycelial growth of all five species examined *in vitro*. Also, chlorothalonil was selected for further study because it completely inhibited spore germination of all the isolates tested at concentrations of 1.25 μg ai ml^{-1} agar. It also caused a decrease in germ tube length of conidia at lower concentrations. It was anticipated that chlorothalonil would be more effective as a pre-inoculation protectant fungicide than a curative product *in vivo*. Also, pyrimethanil was selected for further experiments due to its novel mode of action and because it effectively reduced the diameter of mycelial growth of *Microdochium nivale in vitro*. It is important to note however, the limitations of *in vitro* studies. Fungicides which do not perform well *in vitro* may not be selected for studies *in vivo*, yet they may show activity against the pathogen under glasshouse and field conditions. Hence, this is an obvious limitation of mycelial growth and spore germination studies.

There is limited evidence of fungicidal control of *Fusarium* sp. and *M. nivale in vivo*. Under field conditions, it is difficult to measure the efficacy of fungicides against individual species of

Fusarium and *M. nivale*. Often disease may be caused by several species and the re-isolation of individual species may or may not be effective using conventional techniques. For example, Pettitt *et al.* (1993) showed that conventional media used in the re-isolation of *Fusarium* spp and *Microdochium nivale* from plant material led to an under-estimation of the incidence of *M. nivale*. He therefore recommended that media containing benomyl gave a more realistic estimation of the incidence of *M. nivale*. Also, there may be complex interactions between ear blight pathogens and the naturally occurring ear microflora, such as *Cladosporium* spp., *Alternaria* spp. and *Epicoccum nigrum*. This is discussed in more detail in Chapter 5.

CHAPTER 4

Glasshouse and Field Investigations to Test the Effect of Selected Fungicides and Spray Timing on the Incidence and Severity of Fusarium Ear Blight of Winter Wheat Caused by *F. culmorum* and *M. nivale*, and the Effect on Yield

Introduction

The aim of this study was to investigate the efficacy of selected fungicides against FEB caused by *F. culmorum* and *M. nivale* under glasshouse conditions. Field trials in 1995 and 1996 were also conducted to investigate fungicide effects under field conditions and spray timing on the incidence and severity of FEB caused by *F. culmorum*.

Materials and Methods

Artificial Inoculation of Wheat Ears

The preparation and application of inoculum to wheat ears is described in Chapter 2, (page 34-5). In the first glasshouse experiment, inoculum was derived from three isolates of *F. culmorum* (Fu42, Fu36 and F302, see Table 1, page 33) and was applied when wheat plants were at GS 65. All plants were placed under an overhead mist propagator where mist irrigation of the ears occurred every ten minutes for 15 seconds duration, as determined by a leaf wetness sensor. Plants were misted in this way for two weeks following inoculation.

In the second glasshouse experiment, plants were inoculated with three isolates of *M. nivale* (M15M2, -305/8 and M71, Table 1, page 33) using the same protocol as outlined above for *F. culmorum*. Two inoculations, four days apart, were made to ensure sufficient infection would occur.

In the 1994/95 field trial, plots were inoculated at GS 65 with three isolates of *F. culmorum* (Fu5, Fu60 and Fu42, Table 1, page 33) at a concentration of 100,000 conidia. ml^{-1}. The suspension was applied to all plots at a rate of 33 ml m^{-2}, using a hand-held pressurised sprayer. All plots were mist irrigated following inoculation for three weeks, for approximately ten minutes duration as determined by a leaf wetness sensor which was set-up in plants at ear

height. In the 1995/96 field trial, ears were artificially inoculated at GS 65 with *F. culmorum*, as in 1995, but at a concentration of 200,000 conidia per ml.

Glasshouse Experiments

Tebuconazole, chlorothalonil, prochloraz and pyrimethanil were applied to ears of the winter wheat cv. Avalon at recommended field rate (Table 2, page 36) using the precision pot sprayer. Applications were made either 7 days before, 2 days after or 5 days after the artificial inoculation of ears with *F.culmorum* at GS 65 (see Chapter 2, page 35). Fungicide applications to healthy uninoculated plants allowed potential phytotoxic effects of the fungicides to be determined. Plants which received no fungicide treatment provided untreated controls. Eight pots (5 plants per pot) were allocated for each treatment in a randomised block design.

In a second glasshouse experiment, where ears were artificailly inoculated with *M. nivale*, applications of tebuconazole, chlorothalonil, prochloraz or pyrimethanil were made either five days before, two days after or four days after inoculation.

After the final treatment, and completion of the misting period, plants were removed to a warmer bay of the glasshouse set at 25°C +/- 3°C, with a 12 hour photoperiod, in order to maximise infection. Disease severity was assessed 12 days after inoculation as the percentage of infected spikelets showing symptoms of disease.

Field Trial 1994/95 Experimental Design

Forty plots (12 x 2m) of the cultivar Avalon were sown as a second wheat at Harper Adams College in October 1994 into a sandy loam soil. Agronomy details are listed in Appendix 2.Ten treatments were allocated to plots in a randomised block design, using four replicate blocks.

Treatments comprised of single applications of either prochloraz, tebuconazole, chlorothalonil, pyrimethanil or the biological control agent *Bacillus subtilis* (0.3 g m^{-3} @ 0.15 % w/v, AgrEvo UK Ltd, Hauxton, Cambridge) (see Table 8). All fungicides were applied at recommended field rate (Table 2), either two days before or two days after artificial inoculation of ears. Also included in the design was a formulation of prochloraz and tebuconazole (300 g ai ha^{-1} + 150 g ai ha^{-1}) applied 2 days after inoculation and a two spray treatment consisting of tebuconazole two days pre-inoculation and prochloraz two days post-inoculation. Control plots did not receive any fungicide applications.

Table 8. Fungicides used in 1994/95 field trial

Treatment Number	Fungicide	Time of application
1	prochloraz	2 days before inoculation
2	chlorothalonil	2 days before inoculation
3	tebuconazole	2 days before inoculation
4	prochloraz	2 days after inoculation
5	pyrimethanil	2 days after inoculation
6	tebuconazole	2 days after inoculation
7	tebuconazole	2 days before inoculation
	prochloraz	2 days after inoculation
8	tebuconazole + prochloraz	2 days after inoculation
9	*Bacillus subtilis*	2 days after inoculation
10	Control (untreated)	

Field Trial 1995/96

Eighty four plots (4 x 2m) of winter wheat cultivar Avalon were sown at Harper Adams College in November 1995 in sandy loam soil as a second wheat. Agronomy details are listed

in Appendix 2. Twenty-one treatments were allocated to plots in a randomised block design, using 4 replicate blocks. Prochloraz, pyrimethanil, tebuconazole and copper-prochloraz complex were applied 7, 5, 3 or 1 day prior to inoculation or 1 day post-inoculation with the pathogen. Untreated plots served as inoculated controls. Fungicides were applied at recommended field rate (Table 2). Treatments are listed in Table 9.

Table 9. Treatments used in 1995/96 field trial

Treatment Number	Fungicide	Time of application
1	prochloraz	7 days before inoculation
2	prochloraz	5 days before inoculation
3	prochloraz	3 days before inoculation
4	prochloraz	1 day before inoculation
5	prochloraz	1 day after inoculation
6	pyrimethanil	7 days before inoculation
7	pyrimethanil	5 days before inoculation
8	pyrimethanil	3 days before inoculation
9	pyrimethanil	1 day before inoculation
10	pyrimethanil	1 day after inoculation
11	tebuconazole	7 days before inoculation
12	tebuconazole	5 days before inoculation
13	tebuconazole	3 days before inoculation
14	tebuconazole	1 day before inoculation
15	tebuconazole	1 day after inoculation
16	copper-prochloraz complex	7 days before inoculation
17	copper-prochloraz complex	5 days before inoculation
18	copper-prochloraz complex	3 days before inoculation
19	copper-prochloraz complex	1 day before inoculation
20	copper-prochloraz complex	1 day after inoculation
21	control (untreated)	

Assessment

In the glasshouse experiments, disease severity was recorded as the mean percentage of spikelets showing symptoms of FEB. When ripe, all ears from the glasshouse experiments were harvested and oven-dried for 48 hours at 80°C to constant mass and corrected to 15 % moisture content, before being threshed using a single ear thresher. Total grain weight and the number of grains yielded were recorded for each individual ear along with the thousand grain weight for each treatment.

In the 1994/95 field trial, the incidence (mean percentage of ears infected) and severity (mean percentage of spikelets infected) of FEB was determined at GS 75, 85 and 90 by assessing 100 ears per plot for symptoms of FEB. In 1995/96, 50 ears per plot were assessed at GS 75 and GS 85 for symptoms of FEB. In 1995, on the final assessment date, those ears showing greater than 50 % infection by sooty mould *(Cladosporium herbarum* and *Alternaria alternata*) were recorded to give an indication of the incidence of these secondary colonisers of ears infected with *F. culmorum*.

Total yield and moisture content of grain was recorded for each plot. One hundred gram sub-samples of grain which had been cleaned over a 2 mm sieve were taken from each plot and oven dried to constant mass and adjusted to 15 % moisture, after which time, three replicate thousand grain weights recorded. The total grain weight and moisture content were recorded and the thousand grain weight calculated for each plot.

Statistical Analysis

For all data analysis of variance and T-tests were undertaken. Where incidence and severity data was not normally distributed, angular transformation was carried out.

Results

Glasshouse Experiments

All plants inoculated with either *F. culmorum* or *M. nivale* showed symptoms of FEB, which shows that the inoculation procedure was successful. In the uninoculated plants, some spikelets showed symptoms of FEB, which resulted from cross-contamination from inoculated plants, which was probably due to the dispersal of inoculum from infected plants under the mist irrigation. Re-isolation of *Fusarium* spp. from infected ears, showed that the symptoms were due to infection by *F. culmorum* and *M. nivale* when these species were inoculated. Under glasshouse conditions, tebuconazole and prochloraz consistently gave effective control of FEB caused by *Fusarium culmorum* (Figure 3). Tebuconazole was most efficacious when applied prior to inoculation or up to 2 days after inoculation, reducing the mean percentage of spikelets infected from 30.17 % to 5.06 % when applied 7 days prior to inoculation and to 9.69 % when applied 2 days after inoculation, whereas prochloraz continued to give effective control (reducing disease to 12.61 % and lower) of the disease when applied up to 5 days after inoculation. This shows the protectant and eradicant properties of this fungicide. Compared with tebuconazole, prochloraz performed slightly better when applied after inoculation. Analysis of the yield data showed that only tebuconazole applied 7 days before inoculation caused a significant increase ($p<0.05$) in the thousand grain weight to 31.2 g from 22.5 g for the control. Also, there was a significant increase in total grain weight due to applications of tebuconazole 7 days prior to inoculation from 0.47 g to 0.71 g. Yield data are presented in Appendix 3.

All four products (prochloraz, pyrimethanil, tebuconazole and chlorothalonil) gave effective reduction in FEB caused by *M. nivale* under glasshouse conditions when applied prior to inoculation (Figure 4). There was variable activity of the fungicides when applied after

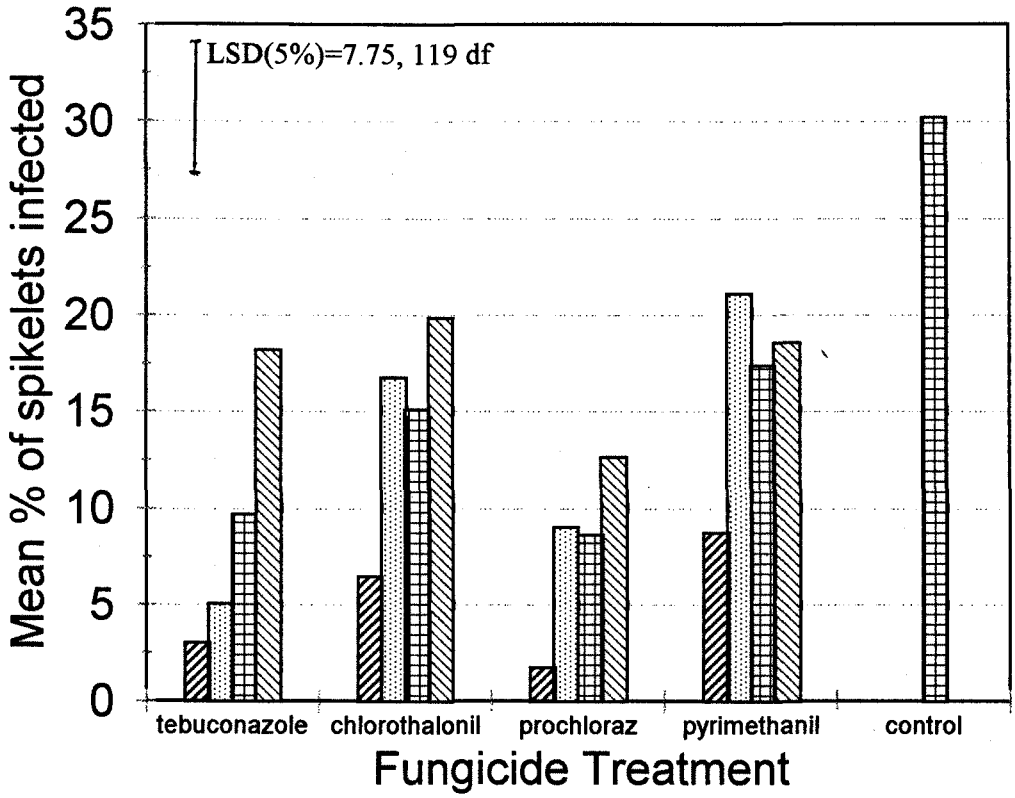


Figure 3. The effect of four fungicides applied either to non-inoculated plants (▨) or 7 days before (▩), 2 days after (▦) and 5 days after (▧) the artificial inoculation of winter wheat ears with *F. culmorum* at GS 65 on the severity of FEB on glasshouse grown wheat plants (cv. Avalon). The LSD bar represents the LSD for treatment / block interaction effects. The control was inoculated but untreated.

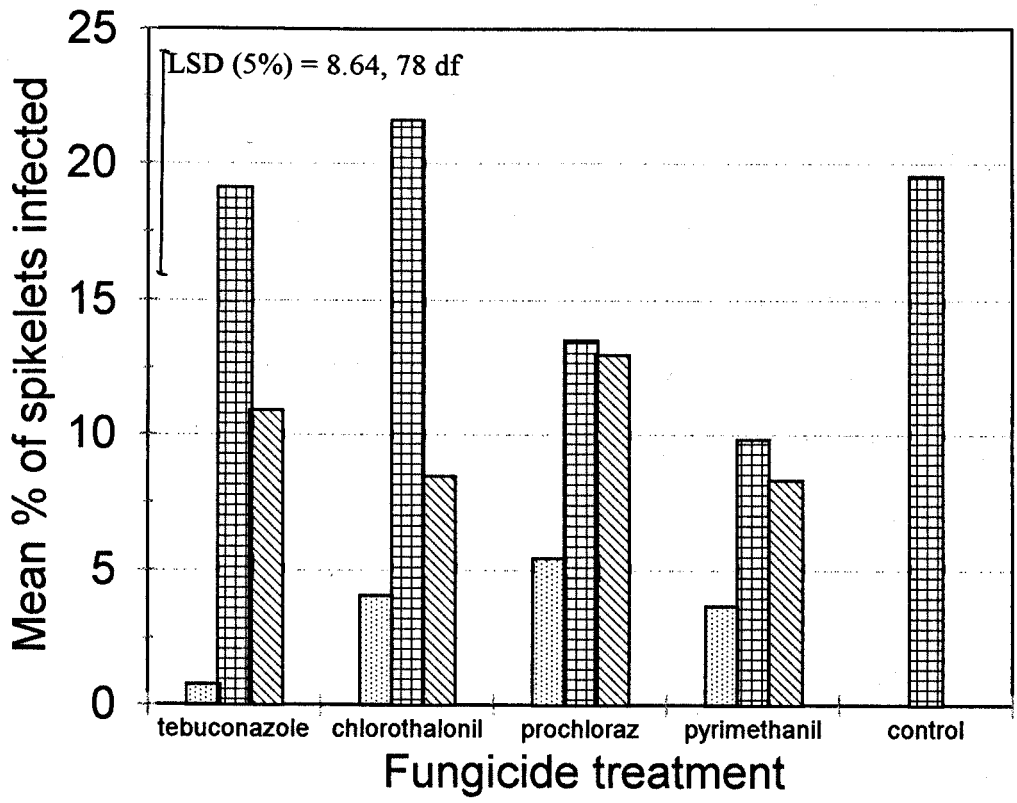


Figure 4. The effect of four fungicides applied 5 days before (▨), 2 days after (▦) and 4 days after (▧) the artificial inoculation of winter wheat ears with *M. nivale* at GS 65 on the severity of FEB on glasshouse grown wheat plants (cv. Avalon). The LSD bar represents the LSD for treatment / block interaction effects. The control was inoculated but untreated.

inoculation. Some reduction in disease was observed with the application of eradicant products applied after infection had occurred, however, it seems likely that the reduction will be less effective and more dependent on the timing of application after infection has occurred. Pyrimethanil showed the best activity, performing consistently well up to 4 days after inoculation. Pyrimethanil showed good protectant and eradicant activity. Disease was reduced from 19.53 % for inoculated control plants to 3.68 % and 9.80 % when pyrimethanil was applied either 5 days before or 2 days after inoculation, respectively. Prochloraz also showed control of the disease, reducing disease to 5.43 %, 13.51 % and 8.30 % when applied 5 days before, 2 and 4 days after inoculation. Only two treatments caused any effect on the yield of wheat. When applied 4 days before inoculation, tebuconazole caused a significant ($p<0.05$) increase in the mean number of grains per ear from 29.8 for the control to 42.2. Pyrimethanil caused an increase in the total grain weight per ear from 1.6 to 2.0 g. Yield data are presented in Appendix 3.

In the 1994/95 field trial, as the season progressed following inoculation, the incidence and severity of Fusarium-infected wheat plants increased. In 1995, all fungicide treatments caused a significant ($p<0.05$) decrease in the severity and incidence of FEB, but the two spray programme which included an application of tebuconazole pre-inoculation and one of prochloraz post-inoculation was the most effective method of reducing the incidence and severity of disease (Tables 10 and 11, respectively). For example, at GS 85, disease severity was reduced from 34.54 % for the control to 16.94 %. The two single most effective treatments were applications of prochloraz two days after inoculation and tebuconazole applied two days before inoculation, which reduced disease severity to 22.36 % and 19.19 % at GS 85, respectively. There was no benefit of mixing these two active ingredients above the performance of the single products alone, as the severity of FEB was reduced to 23.64 %.

Table 10. The effect of selected fungicides when applied before or after the artificial inoculation of ears with *Fusarium culmorum* at mid anthesis (GS 65) on the incidence of FEB (mean percentage of ears affected) recorded at GS 75, 85 and 90 post-artificial inoculation of a field trial in 1995.

			mean % of ears infected		
Treat No.	Fungicide	Time of application	GS 75 5/7/95	GS 85 12/7/95	GS 90 19/7/95
1	prochloraz	2 days before inoculation	19.00	35.67	55.00
2	tebuconazole	2 days before inoculation	24.00	35.33	52.00
3	chlorothalonil	2 days before inoculation	23.00	33.33	55.70
4	prochloraz	2 days after inoculation	29.00	36.67	50.70
5	tebuconazole	2 days after inoculation	30.33	40.00	62.70
6	pyrimethanil	2 days after inoculation	19.00	38.00	59.70
7	tebuconazole	2 days before inoculation	13.33	31.00	58.70
	prochloraz	2 days after inoculation			
8	tebuconazole (150g ai ha^{-1}) +prochloraz (300 g ai ha^{-1})	2 days after inoculation	26.33	36.33	55.30
9	*Bacillus subtilis*	2 days after inoculation	37.00	49.33	72.00
10	control (untreated)	-	32.67	50.33	75.70
LSD (5%)	27 df		11.39	11.68	13.97

Table 11. The effect of selected fungicides when applied before or after the artificial inoculation of ears with *Fusarium culmorum* at mid anthesis (GS 65) on the severity of FEB (mean percentage of spikelets affected) recorded at GS 75, 85 and 90 post-artificial inoculation of a field trial in 1995.

			mean % spikelets infected		
Treat No.	Fungicide	Time of application	GS 75 5/7/95	GS 85 12/7/95	GS 90 19/7/95
1	prochloraz	2 days before inoculation	5.30	21.06	48.79
2	tebuconazole	2 days before inoculation	6.28	19.19	44.08
3	chlorothalonil	2 days before inoculation	6.53	21.14	48.70
4	prochloraz	2 days after inoculation	8.48	22.36	40.96
5	tebuconazole	2 days after inoculation	8.97	24.99	56.00
6	pyrimethanil	2 days after inoculation	3.80	22.26	54.18
7	tebuconazole	2 days before inoculation	3.60	16.94	49.31
	prochloraz	2 days after inoculation			
8	tebuconazole (150g ai ha^{-1}) +prochloraz (300 g ai ha^{-1})	2 days after inoculation	8.29	23.64	48.80
9	*Bacillus subtilis*	2 days after inoculation	14.76	34.07	66.57
10	control (untreated)	-	11.08	34.54	70.59
LSD (5%)	3987 df		2.32	4.86	6.30

Table 12. The effect of selected fungicides when applied before or after the artificial inoculation of ears with *Fusarium culmorum* at mid anthesis (GS 65) on the incidence of sooty mould (*Alternaria* sp. and *Cladosporium* sp.) presented as the mean percentage of ears with greater than 50 % of spikelets infected, recorded at GS 90 (19/7/95) post-artificial inoculation of a field trial in 1995.

Treat No.	Fungicide	Time of application	Incidence of sooty mould at (%) ears >50% infection
1	prochloraz	2 days before inoculation	5.70
2	tebuconazole	2 days before inoculation	12.30
3	chlorothalonil	2 days before inoculation	7.00
4	prochloraz	2 days after inoculation	17.00
5	tebuconazole	2 days after inoculation	7.70
6	pyrimethanil	2 days after inoculation	18.00
7	tebuconazole	2 days before inoculation	6.30
	prochloraz	2 days after inoculation	
8	tebuconazole (150g ai ha^{-1}) +prochloraz (300 g ai ha^{-1})	2 days after inoculation	11.30
9	*Bacillus subtilis*	2 days after inoculation	25.30
10	control (untreated)	-	29.30
LSD (5%)	27 df		11.96

Table 13. The effect of selected fungicides when applied before or after the artificial inoculation of ears with *Fusarium culmorum* at mid anthesis (GS 65) on the severity of FEB (mean percentage of spikelets infected) recorded at GS 75 and 85 post-artificial inoculation of a field trial in 1996.

			mean % spikelets infected	
Treat No.	Fungicide	Time of application	GS 75 13 d after inoculation	GS 85 20 d after inoculation
1	prochloraz	7 days before inoculation	11.38	44.81
2	prochloraz	5 days before inoculation	23.65	62.78
3	prochloraz	3 days before inoculation	11.93	36.55
4	prochloraz	1 days before inoculation	14.59	41.98
5	prochloraz	1 days after inoculation	16.05	49.12
6	pyrimethanil	7 days before inoculation	17.62	52.73
7	pyrimethanil	5 days before inoculation	16.89	50.60
8	pyrimethanil	3 days before inoculation	17.30	48.81
9	pyrimethanil	1 days before inoculation	11.74	47.78
10	pyrimethanil	1 days after inoculation	9.12	37.90
11	tebuconazole	7 days before inoculation	8.07	35.03
12	tebuconazole	5 days before inoculation	10.58	39.08
13	tebuconazole	3 days before inoculation	12.03	43.57
14	tebuconazole	1 days before inoculation	9.00	32.54
15	tebuconazole	1 days after inoculation	15.95	44.49
16	Cu-prochloraz	7 days before inoculation	13.39	41.19
17	Cu-prochloraz	5 days before inoculation	9.87	41.52
18	Cu-prochloraz	3 days before inoculation	10.86	42.70
19	Cu-prochloraz	1 days before inoculation	12.53	43.94
20	Cu-prochloraz	1 days after inoculation	8.61	37.79
21	control (untreated)	-	27.34	59.84
LSD (5%)	4176 df		3.42	7.15

Table 14. The effect of selected fungicides when applied before or after the artificial inoculation of ears with *Fusarium culmorum* at mid anthesis (GS 65) on the incidence of FEB (mean percentage of ears infected) recorded at GS 75 and 85 post-artificial inoculation of a field trial in 1996.

			mean % ears infected	
Treat No.	Fungicide	Time of application	GS 75 13 d after inoculation	GS 85 20 d after inoculation
1	prochloraz	7 days before inoculation	72.00	85.00
2	prochloraz	5 days before inoculation	87.50	92.00
3	prochloraz	3 days before inoculation	64.00	88.50
4	prochloraz	1 days before inoculation	70.00	83.00
5	prochloraz	1 days after inoculation	74.50	86.00
6	pyrimethanil	7 days before inoculation	76.00	86.50
7	pyrimethanil	5 days before inoculation	74.00	89.50
8	pyrimethanil	3 days before inoculation	76.50	86.50
9	pyrimethanil	1 days before inoculation	68.50	90.50
10	pyrimethanil	1 days after inoculation	66.00	85.50
11	tebuconazole	7 days before inoculation	67.50	84.00
12	tebuconazole	5 days before inoculation	68.50	82.00
13	tebuconazole	3 days before inoculation	72.50	84.00
14	tebuconazole	1 days before inoculation	69.50	86.50
15	tebuconazole	1 days after inoculation	72.00	86.50
16	Cu-prochloraz	7 days before inoculation	72.50	81.50
17	Cu-prochloraz	5 days before inoculation	60.00	89.00
18	Cu-prochloraz	3 days before inoculation	60.00	84.00
19	Cu-prochloraz	1 days before inoculation	68.50	81.50
20	Cu-prochloraz	1 days after inoculation	68.00	78.50
21	control (untreated)	-	83.00	89.50
LSD (5%)	60 df		19.91	10.42

There was no significant difference between the single treatments when applied before or after inoculation. The biological control agent *Bacillus subtilis* failed to give any significant decrease in disease. The incidence of sooty mould is shown in Table 12. There was no increase in yield associated with fungicide application. Data for yield are presented in Appendix 3.

In 1996, there was a significant ($p<0.05$) effect of fungicide application on the severity of FEB (Table 13). There was no significant difference between timing of application on the severity of FEB. There was no significant difference between treatments on the incidence of FEB (Table 14). On the later assessment date, when the severity of FEB was greater, some of the fungicides applied seven and five days prior to inoculation failed to cause a significant reduction of ear blight severity. Again, tebuconazole and prochloraz (Copper complex) were the most efficacious products in controlling FEB. The copper complex of prochloraz behaved more consistently at all application timings than the other products. Disease severity at GS 75 was reduced from 27.34 % to between 8.61 % and 13.39 %, irrespective of time of application. Prochloraz and tebuconazole in general performed better when applied prior to inoculation. For example, prochloraz reduced disease severity at GS 75 to 11.38 % and tebuconazole reduced severity to 8.07 % when applied 7 days before inoculation. There was reduced significance between treatments on the second assessment date, although the differences were probably masked by the extreme severity of disease; many ears were approaching 100% of spikelets infected. Indeed, the effect of fungicides on FEB was only just significantly lower than the controls. This suggests that under severe disease pressure the fungicides may be having very little effect on the severity of FEB. Due to weather conditions during June and July 1996, the window of opportunity for spray application could not be extended to include a wider range of post-inoculation treatments, although ideally, the treatments should have been applied 3, 5 and 7 days post-inoculation in addition to the timings investigated above. Obviously, had the

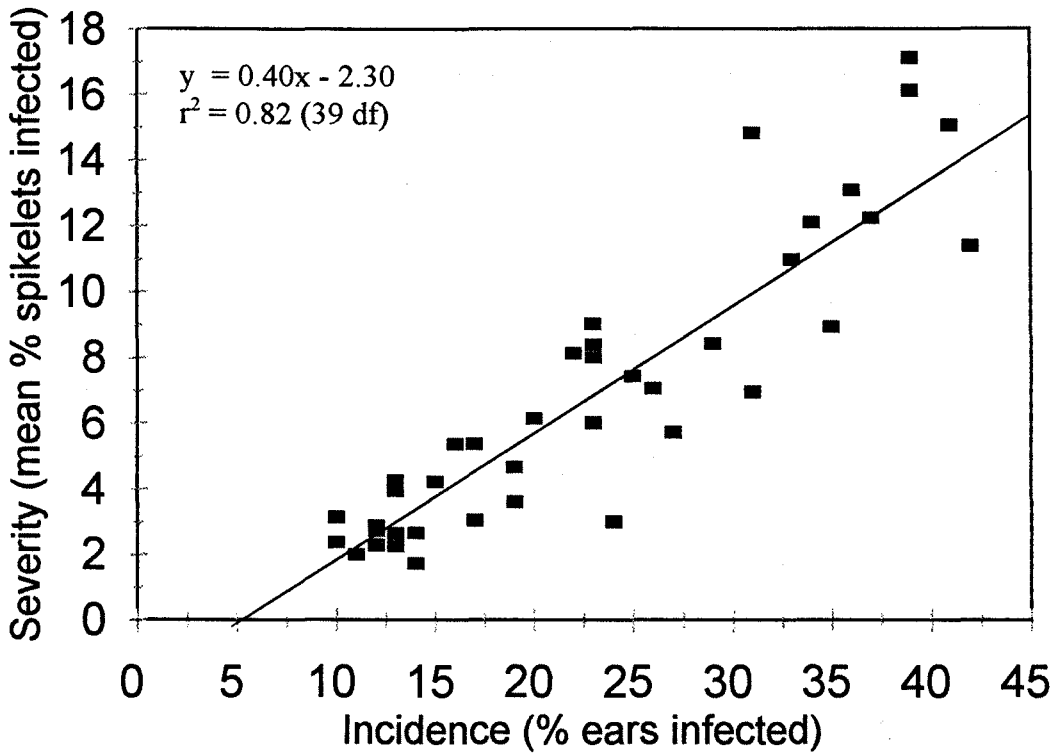


Figure 5. The relationship between incidence (% ears infected) and severity (% spikelets infected) of FEB of winter wheat (cultivar Avalon) on 5/7/95 (GS 75).

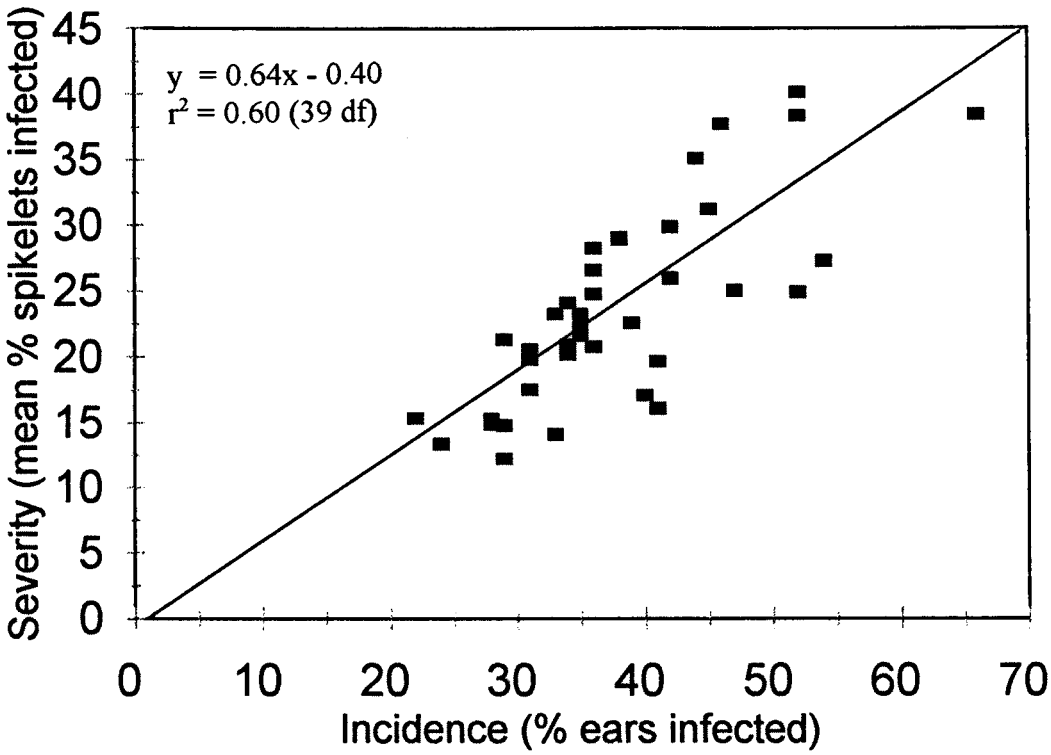


Figure 6. The relationship between incidence (% ears infected) and severity (% spikelets infected) of FEB of winter wheat (cultivar Avalon) on 12/7/95 (GS 85).

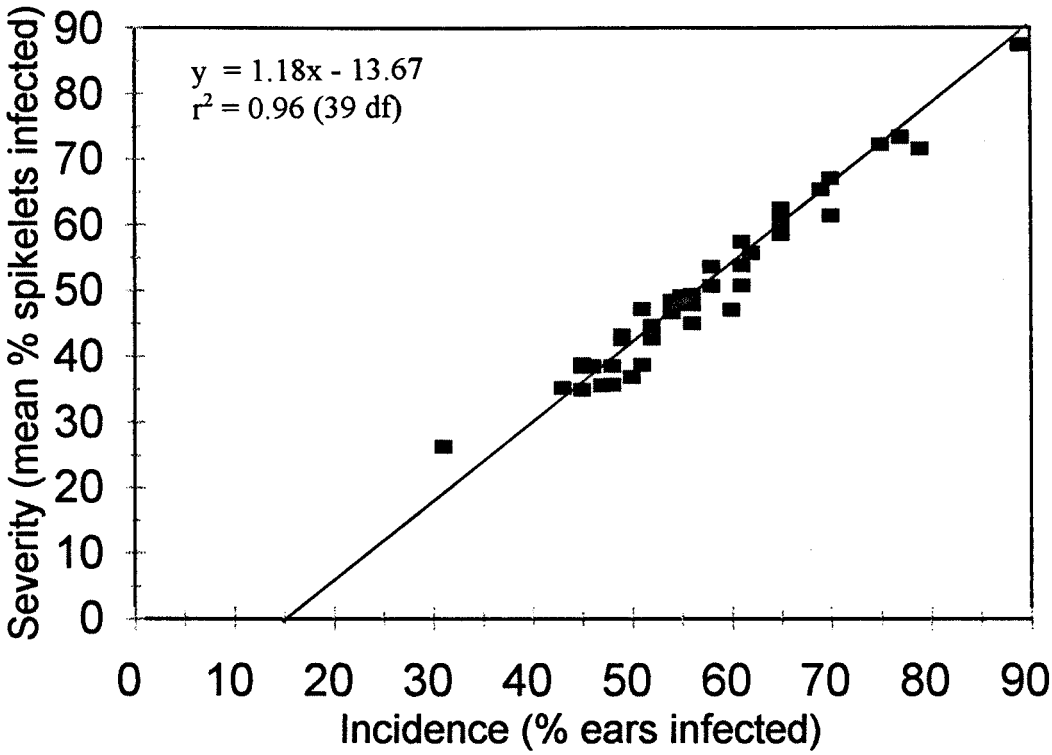


Figure 7. The relationship between incidence (% ears infected) and severity (% spikelets infected) of FEB of winter wheat (cultivar Avalon) on 19/7/95 (GS 90).

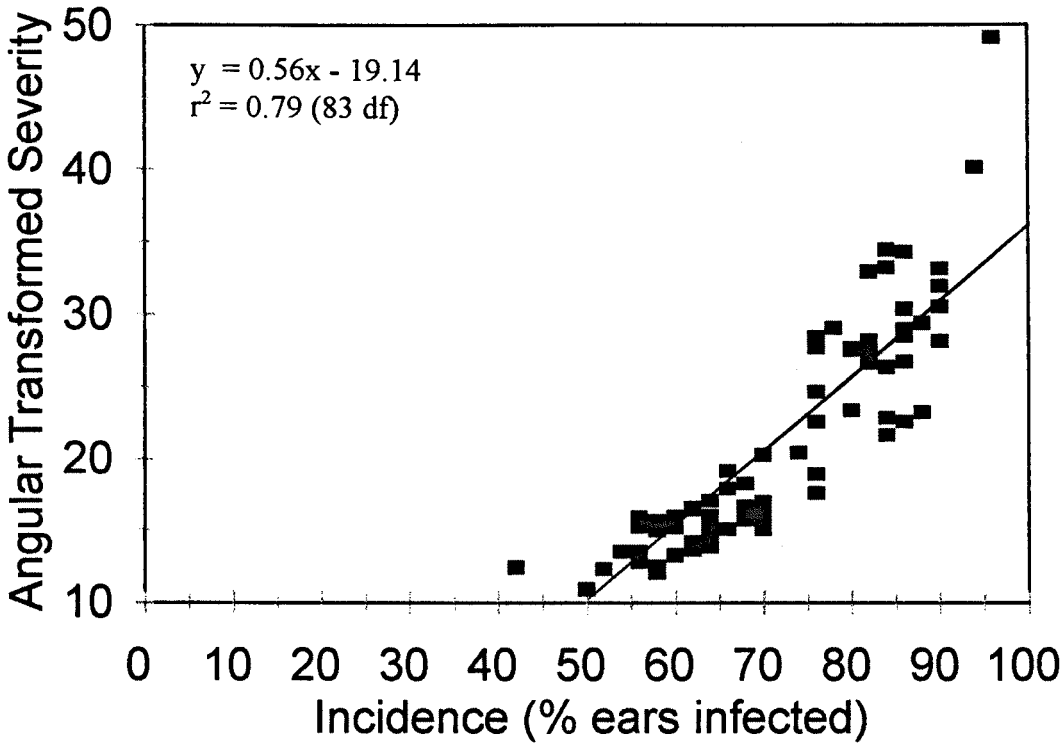


Figure 8. The relationship between incidence (% ears infected) and angular transformed severity (% spikelets infected) of FEB of winter wheat (cultivar Avalon) 20 days after inoculation (GS 85).

weather been more favourable for spray application, a clearer picture would have emerged relating to the most appropriate time for fungicide application to control FEB. No effect of fungicide application on yield and thousand grain weight were recorded. Due to the severe lodging, harvest was difficult and estimates of yield were very low. Data for yield are presented in Appendix 4.

Relationships between disease incidence, severity, yield and thousand grain weight

Regression analysis showed that there were significant ($p< 0.05$) relationships between the incidence and severity of Fusarium ear blight for all three assessment dates in the 1995 field trial. These relationships are illustrated in Figures 5, 6 and 7, respectively. There was also a significant relationship between the incidence and the angular transformation of severity of FEB recorded in the 1996 field trial, at GS 75 (Figure 8). No significant relationship was observed between incidence and severity of FEB at GS 85. This may have been due to the severe lodging which occurred in plots at this time. There was no significant relationship between the incidence or severity of FEB and the incidence of sooty mould in 1995. For both 1995 and 1996 regression analysis failed to reveal any significant relationship between either FEB incidence or severity and yield or FEB and thousand grain weight.

Discussion

In the glasshouse, all plants inoculated with either *F. culmorum* or *M. nivale* showed symptoms of FEB approximately three weeks after inoculation. This shows that the inoculum production and inoculation procedures were successful. Plants which were treated with fungicide but not inoculated showed no phytotoxic effects, however, a small percentage of spikelets showed symptoms of FEB. To ensure that the bleached spikelets were due to infection by *Fusarium culmorum* and not due to phytotoxic effects, the spikelets were surface sterilised and plated onto fungicide amended PDA. Infection of uninoculated plants could be expected to a very limited extent due to the high inoculum present in the glasshouse and misting which produced conditions conducive to infection by the pathogen.

According to Hassall (1990), prochloraz and pyrimethanil are weakly systemic, therefore it could be expected that the time of application of these products would be critical in determining the ability of these products to control FEB. However, Hassall (1990) considered tebuconazole as more systemic. It might, therefore, be expected that timing is less critical in the application and efficacy of this product. There was no increase in yield associated with fungicide application, although, the drought conditions in June and July 1995 and the wet weather and lodging in July and August 1996 which made harvest difficult, may have negated any effect.

The incidence of sooty mould on the ears shows the importance of these saprophytes as secondary colonisers of the ear after the spikelets have been bleached by infection by the ear blight pathogens. It is possible that fungicides which suppress FEB may also reduce the incidence of ear infection by secondary colonisers such as *Alternaria* spp. and *Cladosporium* spp.

Efficacy trials of fungicides for FEB by other workers have been inconclusive. Milus and Parsons (1994) applied tebuconazole to a field trial inoculated with *F. graminearum* and recorded no significant reduction in FEB incidence or increase in yield/test weight which contrasts with data presented here for *F. culmorum*. It is possible that tebuconazole has more activity against *F. culmorum* than *F. graminearum.* In contrast, Suty and Mauler-Machnik (1996) showed that applications of tebuconazole (1 l ha^{-1}) at either GS 55 or GS 65 led to a decrease in the incidence of FEB caused by *Gibberella zeae* (the perfect stage of *F. graminearum*) by over 50 % in a naturally infected field trial. This is in agreement with both glasshouse and field studies presented above for *F. culmorum*.

Work by other authors confirms the lack of reliability of fungicides in controlling FEB. Jacobsen (1977) found that applications of benomyl alone or in combination with mancozeb at GS73 led to a decrease in the incidence of scab caused by *F. graminearum*. The disease was reduced to 4 % from 24.3 % for untreated control plots which shows that application of fungicides at a late stage of development can have beneficial effects on reducing the severity of FEB. Fehrmann and Ahrens (1985) also showed that an application of prochloraz (1.2 l ha^{-1}) led to an increase in yield when applied to plants infected with *F. culmorum, F. graminearum* and *S. nodorum*, although no disease data was presented to confirm that the yield increase was associated with a reduction in the severity of disease. Martin and Johnston (1982) showed that applications of propiconazole at GS 50 and GS 75 caused a decrease in the incidence of FEB of 41 % with a concurrent yield increase of 34 %. The yield increase may have been caused by a reduction in the saprophytic microflora of wheat which may normally cause premature senescence of wheat leaves (Skidmore and Dickinson, 1973). Also, other fungal pathogens such as *Septoria* spp may have been controlled by the fungicides (Jacobsen, 1977). Although, there was a significant reduction in FEB due to fungicide application in the 1995-96 trial at Harper

Adams, certain fungicides caused an increase in yield although this was not a statistically significant increase. Hence, the relationship between FEB severity and yield is a complex and unpredictable one. As a result, fungicide applications which reduce the severity of disease may not necessarily be directly responsible for a yield increase. Furthermore, fungicide application may have a profound effect on the mycotoxin contamination of grain. There is experimental evidence to suggest that applications of fungicides may decrease mycotoxin production by the pathogen. For example, although Boyacioglu *et al.* (1992) showed that thiabendazole failed to reduce the incidence of FEB in field trials inoculated with *F. graminearum*, however, it caused a significant reduction in the concentration of DON in the grain by 83.4 % was recorded following applications of the fungicide. Hence, grain quality in addition to yield is an important factor to consider when applying fungicides to control ear blight pathogens. The study of mycotoxin production is a huge task in itself and is beyond the scope of this project.

Other factors which would have an effect on fungicide efficacy against ear blight pathogens include the availability of inoculum. For example, according to Cook (1980), infected crop debris present on the surface of soil provides a primary source of inoculum for the development of FEB epidemics. Hence, the amount of inoculum and combination of species may vary from site to site, particularly where trials rely on natural inoculum. This may explain the discrepancy between the results for fungicide trials reported by different workers. The colonisation of ears by saprophytic microflora may also affect fungicide efficacy under field and glasshouse conditions. The presence of saprophytic microflora such as *Alternaria alternata, Aspergillus niger, Botrytis cinerea, Cladosporium* spp and *Epicoccum nigrum* isolated from wheat ears (Grabarkiewicz-Szczęsna *et al.*, 1989) may compete with *Fusarium* species on the ears of wheat. Subsequent inhibition of the microflora by fungicides may then lead to occupancy of the niche by a pathogenic *Fusarium* species (Dik, 1992). The relationship between *Fusarium*

culmorum and *Microdochium nivale* with the saprophytic microflora species *A. alternata, B. cinerea* and *C. herbarum* are discussed in detail in Chapter 5 and the relationship between these species and selected fungicides is discussed in Chapter 6.

The conditions immediately after fungicide application have an important effect on fungicide adhesion, penetration and therefore, activity. For example, triazoles such as tebuconazole, flusilazole and flutriafol, are less active under cool and humid conditions (Gisi *et al.*, 1986). Also, light rain or dew may lead to redistribution of fungicides to or from the site of infection by the pathogen. For example, Cooke *et al.*, 1989 showed that prochloraz was redistributed on the foliar surfaces of winter wheat plants following simulated rainfall. The re-distribution was proportional to the extent of the rainfall. Also, the cultivar of wheat and the formulation of the fungicide may affect the fungicide adhesion and retention and therefore its activity against a plant pathogen. These factors are discussed in Chapter 7.

CHAPTER 5

The Role of Saprophytic Microflora in the Development of Fusarium Ear Blight of Winter Wheat Caused by *Fusarium culmorum* and *Microdochium nivale*

Introduction

The discrepancy between fungicide performance *in vitro* and *in vivo* may be explained by several factors including application and formulation of products. In addition, the presence of saprophytic fungi such as *Alternaria alternata, Botrytis cinerea, Cladosporium herbarum, Epicoccum nigrum* and *Aspergillus niger* which have been isolated from wheat ears (Grabarkiewicz-Szczęsna *et al.*, 1989) may also influence field performance of fungicides. Dik (1992), whilst reviewing the interactions among fungicides, pathogens, yeasts and nutrients in the phyllosphere, suggested that a reduction in saprophyte populations by fungicides may decrease their antagonistic effect against pathogens. In the case of competition for nutrients, this would affect the amount of nutrients in the phyllosphere increasing that available for pathogens to exploit.

The disease of wheat grain, blackpoint, is caused by members of the Deuteromycotina division and class Hyphomycetes of fungi. Symptoms occur on ripening cereal ears. Grey-black superficial mycelium develops on the outer glumes and there may be darkening and shrivelling of the embryo end of the grain (Lorenz, 1986). These fungi produce dark pigmented asexual conidia which are dispersed by wind. *Alternaria alternata* is the most common fungus causing blackpoint, although other species are involved including *C. herbarum*. According to Lorenz (1986), the disease commonly develops during the ripening of grain. Using scanning electron microscopy, it was shown that mycelial growth occurred throughout the embryo and within the pericarp layer along the crease region of infected grain, but the fungi did not invade the endosperm. The plumpest grain were the worst affected and it was suggested that they matured earlier on the primary tillers and might have been invaded by the fungus during the wetter part of the growing season.

King *et al.* (1981) investigated the brown/black discolouration and shrivelling of wheat grain. Whilst isolating fungi growing from dicoloured grain, these workers showed that *Alternaria* spp. were isolated from 89 % of diseased grain but *Botrytis cinerea* was only recovered from 27 % of grains. Although *B.cinerea* was not commonly isolated from grain its occurrence on leaves and glumes had increased over a number of years and it is thought that this *Botyrtis* contamination of flour is a potential problem to millers. Grains from the single *Cladosporium* infected sample had a bleached appearance compared to the blackened kernels infected with *Alternaria* spp.

In 1986 and 1987 in Poland, Grabarkiewicz-Szczesna *et al.* (1989) sampled discoloured rye and wheat ears to identify the fungal genera present. *Cladosporium* spp., *Alternaria* spp., *Drechslera sorokiniana* and *Epicoccum purpurascens* were identified microscopically in washings from ears. Analysis of the mycotoxins in the chaff showed that 10 % of wheat samples were contaminated with up to 1.8 mg kg^{-1} alternariol and 19 % of wheat samples were contaminated with up to 0.51 mg kg^{-1} alternariol methyl ester.

A study of seed samples of *Triticum* spp. in New Delhi showed that *Microdochium nivale, Botrytis cinerea, Drechslera sorokiniana, F. culmorum* and *F. poae* were the common contaminants (Dev, 1989). Clear and Patrick, (1993), whilst undertaking a survey of seed-borne fungi from wheat seed harvested from Ontario, Canada, isolated 59 species representing 35 fungal genera between 1988 to 1990. The microflora isolated included *A. alternata, Epicoccum nigrum* and species of *Arthrinium, Aspergillus, Cladosporium, Drechslera, Fusarium* and *Septoria.* In any one year, *Alternaria alternata* occurred in up to 98 % of samples, *Aspergillus* spp. in up to 69 %, *Cladosporium* spp. in up to 23 %, *Epicoccum nigrum* in up to 25% and *F. graminearum* in up to 85 %. The other fungi occurred in less than 20 % of samples in any

year.

Jachmann and Fehrmann, (1989) found that when plants were inoculated with *C. herbarum* and *C. cladosporoides*, the chlorophyll content of the leaves was reduced by up to 70 % compared to untreated plants, however inoculations of *Epicoccum purpurascens*, *A.alternata* and white yeasts did not promote premature senescence.

There has been little research to understand the possible interactions between cereal ear pathogens, saprophytic microflora of wheat and fungicides. These relationships may be fundamental to the understanding of disease development and management. Indeed, the control measures which are employed may have a profound impact on the natural microflora of plants, microflora which may have a suppressive or antagonistic effect on plant pathogens. For example, Bateman (1979) observed the relationship between saprophytes and *M. nivale* on wheat seed, and suggested that removal of an antagonistic organism by a dose of organomercury fungicide, insufficient to affect *M. nivale*, might increase disease. He also found that ears inoculated with *Alternaria*, *Cladosporium* and/or *Sporobolomyces* at anthesis, 13 days following inoculation with *M. nivale* led to reduced recovery of *M. nivale* from the seed. Inoculations made directly onto the seed showed also that *Alternaria* was the only saprophyte which significantly reduced the severity of seed infection by *M. nivale*.

A number of studies have been undertaken to determine the effect of fungicides on the phyllosphere and ear microflora of crops in order to establish a relationship between pathogens, epiphytic microflora and the fungicides used to control plant pathogens. For example, *B. cinerea* was shown to reduce the severity of leaf spot caused by *Helminthosporium* spp. by 47.5 % under glasshouse conditions (Bansal *et al.*, 1988). Many of the experiments have

studied the leaf surface and its associated phyllosphere, however, there have been limited studies to relate saprophytic ear microflora to Fusarium ear blight pathogens. Harris *et al.* (1979) found that in addition to the control of foliar and stem diseases of cereals such as eyespot (*Pseudocercosporella herpotrichoides*), leaf blotch (*Septoria tritici*) and powdery mildew (*Erysiphe graminis*), prochloraz also controlled ear diseases such as glume blotch (*Septoria nodorum*) and Fusarium ear blight and the black and sooty moulds (*Alternaria* spp. and *Cladosporium* spp.). In a field trial in Austria, Harris *et al.* (1979) noted a decrease in the severity of black and sooty moulds and ear mildew, although no disease data were provided. They demonstrated an increase in yield of 7 % resulting from applications of prochloraz at a rate of 500g a.i ha^{-1} and 10 % due to applications of prochloraz at 750g a.i ha^{-1}. It is however, unwise to assume that the increase in yield as a result of fungicide application was due to a decrease in the severity of disease, in the absence of disease data.

Meunier and Meyer (1985) also investigated the effects of fungicide treatments on the epiphytic microflora on the leaves of winter wheat. The cultivars Armada and Fidel were treated with fungicides at ear emergence. Seven days after treatment, the applications of chlorothalonil (Daconil 500, 1000 g ai ha^{-1}), maneb (Polygram M BASF, 1600 g ai ha^{-1}), propiconazole (Tilt, 125 g ai ha^{-1}) and the mixtures fenpropimorph plus chlorothalonil (Corbel Star, 600 g ai ha^{-1} plus 999 g ai ha^{-1}), and triadimefon plus carbendazim plus captafol (Bayleton Triple, 125 g ai ha^{-1} + 800 g ai ha^{-1} + 200 g ai ha^{-1}) all decreased the populations of bacteria from 1.72 x 10^2 per cm^2 leaf to between 0 and 0.23 x 10^2 per cm^2 leaf, the yeasts from 7.76 x 10^2 per cm^2 to between 0.29 to 1.13 x 10^2 per cm^2 and fungi from 0.57 x 10^2 per cm^2 to between 0.06 and 0.33 x 10^2 per cm^2 after 7 days. The predominant species isolated were pink and white yeasts, the bacteria were *Pseudomonas* spp. and the fungi were *Cladosporium* spp., *Aureobasidium pullulans*, *Fusarium* spp. and *Alternaria* spp. The effect of the fungicides on individual species

of yeast and fungi was not recorded.

Magan and Lacey (1986) found that the mycoflora colonising the winter wheat cultivar Maris Huntsman and spring wheat cultivar Timmo included *Cladosporium* spp., *Verticillium lecanii, Alternaria alternata, Fusarium* spp. and *Epicoccum nigrum*. In general, the numbers (log number of colony forming units re-isolated using dilution plating) of these fungi increased on the flag leaf and ears between anthesis and harvest. It was shown that captafol applied at either GS 38-40 or GS 50-60 acted as an effective protectant, decreasing the total population of yeasts and filamentous fungi on flag leaves for four weeks. The numbers of yeasts and fungi were determined as the $\log_e$+0.5 colonies recovered per gram fresh weight and varied between 9.89 and 10.56 for assessments made at GS 50, 66 and 70 which was significantly lower than the untreated which varied between 12.88 and 13.37 for the 4 week period covering these growth stages. Also, benomyl and maneb were effective against yeast-like fungi and *Cladosporium* spp. In general, captafol decreased the numbers of *Aureobasidium pullulans, Hyalodendron* spp., yeasts, *Cladosporium* spp., *Verticillium lecanii, Epicoccum nigrum* and *Fusarium* spp. more than any other fungicide, although no fungicide effectively controlled *A. alternata.* For example, in one season, the total number of yeasts, mycelial yeasts and filamentous fungi was reduced from 2.82×10^{10} per gram fresh weight for untreated plots to 2.82×10^{7} per gram for captafol treated plots on 23 June 1981. No exact figures for individual species were presented. According to Magan and Lacey (1986), application of captafol gave an increased yield as a result of increased 1000 grain weight due to a combination of delayed leaf senescence and control of surface microflora, however, it is difficult to identify which factor gave the most significant effect on yield. Early applications of fungicides at GS 38-40 failed to reduce the severity of *Septoria* leaf spot (*Septoria tritici*), brown rust (*Puccinia recondita*) and powdery mildew (*Erysiphe graminis*), hence the yield increase due to fungicide

application was not attributed to control of these diseases.

The sensitivity of phylloplane organisms to fungicides *in vitro* was also investigated by Smiley *et al.*, (1993). *Cladosporium macrocarpum* and *C. herbarum* were highly sensitive to prochloraz and propiconazole, showing ED_{100} values of less than 1µg ml^{-1}. These organisms showed moderate sensitivity to chlorothalonil, fenarimol and triadimefon (ED_{100}<10µg ml^{-1}, ED_{50}<1 µg ml^{-1}). They were sensitive to mancozeb (ED_{50} >1µg ml^{-1}, ED_{100} >10µg ml^{-1}), moderately tolerant of benomyl (ED_{50}<1µg.ml^{-1}, ED_{100}>10µg.ml^{-1}) and tolerant of flutolanil and tolclofos-methyl (ED_{50}>1µg.ml^{-1}, ED_{100}>10µg.ml^{-1}). No formulation details for the fungicides were provided, therefore, direct comparisons with data presented by other authors cannot be made. *Cladosporium cladosporioides* was not highly sensitive to any fungicide but showed moderate sensitivity to fenarimol, prochloraz and propiconazole, moderate tolerance to tolclofos-methyl and tolerance to benomyl, chlorothalonil, flutolanil, mancozeb and triadimefon. *Alternaria* spp. were moderately tolerant of fenarimol, prochloraz, propiconazole and tolclofos-methyl and tolerant of benomyl, chlorothalonil, flutolanil, mancozeb and triadimefon. When fungicides were applied to the flag leaf in the field under natural infection conditions, a single application of any product failed to suppress leaf spot caused by *Cladosporium* spp. or increase yield. When multiple applications were made, leaf spot incidence on the second leaf was significantly reduced by benomyl, mancozeb, propiconazole, sulphur or copper sulphate. No product reduced the incidence on the third leaf. There was no significant relationship between incidence and severity. Only benomyl gave an improved yield, however, this was unrelated to disease suppression and may have resulted from delayed leaf senescence or a stimulatory effect on plant growth and production.

There is evidence from fungicide trial experiments that fungicides affect saprophytic microflora

in addition to plant pathogens and this may complicate the interpretation of fungicide efficacy experiments. For example, research by Wainwright *et al.* (1992) using tebuconazole, has shown how difficult it is to interpret fungicide trial data. Tebuconazole applied to winter wheat at 1 l ha^{-1} at GS 59 resulted in a reduction in the spore counts of a number of fungal species on senescing leaves and ears. Ears of the winter wheat cultivar Avalon were washed and spore counts measured. The spore count for *Cladosporium* spp was reduced from 94 spores per ml (untreated) to 40 spores per ml after application of tebuconazole. The count for *Alternaria* spp. was reduced from 67 to 21. Spore counts for *Fusarium* spp., were reduced from 20 for controls to 16 after treatment with tebuconazole plus triadimenol (1 l ha^{-1}), although this was unlikely to have been a significant reduction. Spore counts of *Septoria nodorum, Botrytis cinerea* and *Septoria tritici* were also reduced by these treatments. It is likely that the 'brighter ears' observed by Wainwright *et al.* (1992) were due to an overall reduction in ear microflora including saprophytes and pathogens. It was also suggested that prolonged presence of green tissue due to delayed senescence also contributed to 'brighter ears' (lower densities of fungi with blackened hyphae and conidia) although further research is required to confirm this.

Dik (1992), whilst reviewing the interactions among fungicides, pathogens, yeasts and nutrients in the phyllosphere, suggested that in addition to the theory that fungicide application may increase yield by reducing the populations of saprophytic microflora, causing decreased senescence, a reduction in saprophytes by fungicides may decrease their antagonistic effect against pathogens. In the case of competition for nutrients, this would affect the amount of nutrients in the phyllosphere, increasing that available for pathogens to exploit.

Although saprophytic fungi may interact with ear blight pathogens on the ears of wheat, there is little experimental evidence which demonstrates that such interactions could account for the

poor performance of fungicides *in vivo* against FEB. The aim of these studies was to evaluate the role of *A. alternata, B. cinerea* and *C. herbarum* in the development of FEB symptoms caused by *F. culmorum* using glasshouse and *in vitro* competition experiments. The effect of fungicides on saprophytic microflora is discussed in Chapter 6.

Materials and methods

Glasshouse Experiments

The preparation of winter wheat (cultivar Avalon) plants is described in Chapter 2, page 35. Inoculation of the winter wheat cultivar Avalon with three isolates of *F. culmorum* (Fu42, Fu36, F200, Table 1) was undertaken at mid anthesis (GS 65). Inoculations of either *A. alternata, B. cinerea* or *C. herbarum* occurred either when ear emergence was complete (GS 59) or when anthesis was complete (GS 69). Spore concentrations of 200,000 spores per ml were used and ears were sprayed until run-off using a hand-held atomiser (approximately 2 ml per ear). Eight replicate pots were set-up for each treatment and arranged in a randomised block design. Following inoculation, the ears were individually covered with self-sealing clear polythene bags for 48 hours to provide conditions conducive to ear infection. At GS 70 and GS 80, all ears were assessed for the severity of FEB symptoms. The total number of spikelets and the number showing necrosis or bleaching was recorded to give the percentage of infected spikelets. In a second experiment, wheat plants were inoculated with *M. nivale* (isolates M15M2, M58, M71 and RFN_2I) instead of *F. culmorum*.

In vitro studies

Determination of inhibition of pathogen growth

Dual cultures were used to investigate the relationship between saprophytic microflora and the pathogens *F. culmorum* (Fu42, Fu36, F200 and F302, Table 1) and *M. nivale* (M15M2, M58,

M71 and RFN_2I, Table 1). Individual isolates of *A. alternata*, *B. cinerea* and *C. herbarum* were each grown in dual culture with four isolates of *F. culmorum* and four isolates of *M. nivale* according to the method described by Dennis and Webster (1971a). For each of the three saprophytic species, a 5 mm plug, taken from an actively growing colony, was placed onto a 9 cm plastic Petri dish containing PDA either 4, 3, 2 or 1 day before the introduction of a plug of the pathogen to determine whether a delay in the introduction of *F. culmorum* or *M. nivale* led to a greater antagonistic effect of the saprophyte. Saprophyte and pathogen plugs were placed 3cm apart. All plates were sealed with parafilm and incubated at 20°C for 7 days. Five replicate plates were prepared for each treatment. After incubation, the diameters of all pathogen colonies were measured and any discrete zone of inhibition observed between saprophyte and pathogen colonies was recorded. The experiments were repeated and the results comparable.

Determination of non-volatile antibiosis

The method for this experiment was suggested by Preece, pers comm. To determine whether there was non-volatile antibiotic production by the saprophytic microflora, for each saprophyte, 5 mm plugs were taken from an actively growing colony and placed centrally onto separate plates of PDA onto which cellophane discs had been placed. Plates were incubated at 20°C for either 4, 3, 2 or 1 day. Following incubation, the cellophane and colonies of saprophytes were removed from the PDA. The plates were then inoculated with 5mm plugs of *F. culmorum* or *M. nivale* and sealed with Parafilm. All plates were re-incubated in the dark at 20°C for a further 4 days after which time the diameters of the pathogen colonies were measured. Five replicate plates were inoculated for each treatment and the experiment was repeated.

Determination of volatile antibiosis

To determine whether the saprophytic species produced volatile antibiotics which suppressed *F. culmorum* or *M. nivale in vitro*, for each saprophyte, 5 mm plugs were taken from an actively growing colony and placed centrally onto separate plates of PDA. Plates were incubated at 20°C for either 4, 3, 2 or 1 day. Following incubation, plates of PDA which had recently been inoculated with 5 mm plugs of *F. culmorum* or *M. nivale* were inverted and placed over each of the saprophytic colonies. Both plates were sealed together using cellophane tape. All plates were re-incubated in the dark at 20°C for 5 days after which the diameter of the pathogen colonies were measured. Five replicate plates were set-up for each treatment. The experiment was repeated. Control treatments consisted of each pathogen isolate inoculated onto plates directly below existing colonies of themselves.

Determination of antimycelial / antisporulant products

To determine whether water soluble extracellular compounds produced by *Alternaria alternata, Botrytis cinerea* and *Cladosporium herbarum* were inhibitory to mycelial growth and spore germination of *Fusarium culmorum* and *Microdochium nivale*, cell free filtrates were used. Liquid culture extracts of *A. alternata, B. cinerea, C. herbarum, F. culmorum* (Fu42 and F200) and *M. nivale* (RFN$_2$I and M15M2) were prepared as follows. Conical flasks (250ml) containing 125 ml of sterile potato dextrose broth (PDB, Sigma Chemical Co., St.Louis, USA) were inoculated with a 15mm diameter plug of each isolate removed from the edges of 7 day old cultures of the isolates. The flasks were stoppered with a sterile cotton wool bungs, covered with aluminium foil and sealed with Parafilm. The flasks were incubated at 20°C +/- 2°C in a rotary shaker (Infors AG, Rittergasse 27, CH-4103, Bottmingen) at 185 rpm for four weeks. To remove the cells, cultures were filtered, first through several layers of sterile muslin, then through two layers of sterile filter paper (No.3, Whatman Labsales Limited, St.Leonards Road, Maidstone, Kent) and finally through disposable sterile micropore filter units (Mesh Size, 0.2

µm, Nalgene (Europe) Ltd, Rotherwas, Hereford) to remove the cells and contaminants. Filtrates were stored at 4°C until required.

The cell free filtrates were tested for antibiosis against mycelial growth of two isolates of *F. culmorum* and two isolates of *M. nivale in vitro*. The isolates were grown on PDA to investigate the mycelial growth when inoculated opposite 5mm diameter wells containing the cell free filtrates. Mycelial plugs measuring 5 mm diameter of the isolates of *F. culmorum* and *M. nivale* were inoculated onto PDA plates 3 cm from wells containing cell free filtrates. Three replicates were assessed for each combination of pathogen isolate and well containing cell free filtrate.

The diameter of pathogen colonies was measured daily. Plates were also assessed for the presence of a zone of inhibition between the mycelial growth of the pathogen and the well. The diameter of each pathogen colony after 4 days was analysed for each isolate using analysis of variance.

The cell free filtrates were also tested for antibiotic activity against spore germination of *F. culmorum* and *M. nivale in vitro*. Three sterile cellophane squares were placed onto the surface of tapwater agar, as described in Chapter 3. Onto these, 25µl of spore suspension (200, 000 conidia per ml) of either *F. culmorum* or *M. nivale* was pipetted with 25µl of a cell free extract from one of the microflora organisms.

Three replicate plates for each saprophyte/pathogen combination were prepared. After 9 hours incubation in an incubator at 22 +/- 2°C, percentage germination was assessed for each cellophane square. One hundred spores were counted per square and spores were considered

to have germinated when the length of germ tube was longer than the width of the spore. Control plates consisted of spore suspensions inoculated onto cellophane squares on the surface of TWA without any cell free filtrate.

Electron Microscope Studies

Scanning Electron Microscopy was used to investigate the morphology of the interaction between the potential antagonists *A. alternata, B. cinerea* and *C. herbarum*, and *F. culmorum in vivo* on the grain and spikelets of winter wheat. Grain of the winter wheat cultivar Avalon was surface sterilised using 5 % sodium hypochlorite for 2 minutes and washed 3 times in sterile distilled water prior to drying on sterile filter paper in a laminar flow. The grain was then soaked in a conidial suspension of either *A. alternata, B. cinerea* or *C. herbarum* (200,000 conidia per ml) in a sterile McCartney tube for 30 minutes. The grain was removed and the inoculum allowed to dry on the grain in the sterile laminar flow unit. The grain was then soaked in a conidial suspension of *F. culmorum* (isolate Fu42) for a further 30 minutes and dried. The grain was then placed on the surface of tap water agar in plastic Petri-dishes and incubated for 24 hours in darkness at 20°C +/-2°C. The samples were fixed and dehydrated by immersion in 3 % glutaraldehyde in 50mM phosphate buffer at pH 7 at 4°C. The samples were then dehydrated in an ethanol and acetone series, critical point dried using a Balzers CPD-020 with carbon dioxide as a transition fluid, and mounted on metal stubs. The tissue was gold coated in an Edwards S 150 sputter coater. Specimens were viewed with a Cambridge Stereoscan 200, operating at 10kV.

Additionally, grain was soaked in the cell free filtrate of either *A. alternata, B. cinerea* or *C. herbarum* for 30 minutes prior to inoculation of the grain with *F. culmorum* to determine whether the extracts had any inhibitory effect on spore germination or hyphal development of

the pathogen *in vivo*.

A similar study was undertaken on the spikelets of wheat (cv. Avalon). Ears at anthesis were removed from glasshouse grown plants and surface sterilised using 5 % sodium hypochlorite for two minutes. The ears were mounted vertically in 'oasis' (artificial medium used to support flowers in arrangements) and dried in the laminar flow following rinsing in sterile distilled water. When dry, a conidial suspension of either *A. alternata, B. cinerea* or *C. herbarum* (200,000 conidia per ml) was applied to 3 replicate ears using a hand-held atomiser. The ears were sprayed until run-off (approximately 2ml per ear) and then placed in self-sealing plastic bags. The ears were incubated overnight at 20°C +/-2°C in darkness. The ears were removed from the bags and inoculated with a spore suspension of *F. culmorum* (200,000 conidia per ml) until run-off. The ears were returned to the bags for a further 24 hours incubation and then spikelets removed from the middle of the ear for fixing and dehydration as described earlier.

Results

Glasshouse Experiment

In the glasshouse, symptoms of ear blight were observed on all ears artificially inoculated with *Fusarium culmorum*. Plants inoculated with the saprophytic species alone had no symptoms and plants inoculated with *F. culmorum* alone resulted in 19.1 % of spikelets infected at GS 75 (Table 15). Inoculation of ears with any of the saprophytic species before the introduction of *F. culmorum*, significantly reduced the severity of FEB symptoms to between 4 and 6 %.

The introduction of *A. alternata* after the inoculation of ears with *F. culmorum* significantly increased the severity of FEB symptoms observed when compared to control treatments. No significant effect on disease severity was recorded when *B. cinerea* and *C. herbarum* were

Table 15. Effect of artificially inoculating ears of winter wheat (cv. Avalon) with *Alternaria alternata, Botrytis cinerea* or *Cladosporium herbarum* at GS 59 or GS 69 (Zadoks *et al.*, 1974) on the development of Fusarium ear blight (FEB) symptoms assessed at GS 75 after artificial inoculation of ears with *F.culmorum* at GS 65.

ORGANISM AND TIME OF INOCULATION			% SPIKELETS INFECTED	
GS 59	GS 65	GS 69	GS 75	GS 80
A.alternata			0	0
B.cinerea			0	0
C.herbarum			0	0
	F.culmorum		19.1	26.7
A.alternata	*F.culmorum*		4.5	6.0
B.cinerea	*F.culmorum*		6.7	14.5
C.herbarum	*F.culmorum*		5.3	9.8
	F.culmorum	*A.alternata*	31.1	39.9
	F.culmorum	*B.cinerea*	19.7	25.2
	F.culmorum	*C.herbarum*	24.7	30.0
		LSD (p=5%)	6.1	7.6
		40 df		

ANOVA F probabilities;

GS 75: treatment <0.001
SED (treatment) = 3.036, 8 df

GS 80: treatment <0.001
SED (treatment) = 3.742, 8 df

introduced after the inoculation of ears with *F. culmorum*.

Symptoms of ear blight were observed on all ears artificially inoculated with *M. nivale*. Plants inoculated with *M.nivale* resulted in 7.9 % of spikelets infected at GS 80 (Table 16). Inoculation of ears with either *A. alternata* or *B. cinerea* in addition to *M. nivale* had no significant effect on FEB. However, when plants were inoculated with *C. herbarum* either before or after the inoculation of ears with *M. nivale* there was an increase in the severity of FEB to 17.2 and 16.9 % respectively.

In vitro studies

Determination of inhibition of pathogen growth

In vitro dual culture experiments showed that pre-inoculation of PDA with either *A. alternata, B. cinerea* or *C. herbarum* prior to introduction of *F. culmorum* resulted in a significant ($p<0.05$) decrease in the colony diameter of the pathogen (Figure 9). For example, in the absence of any saprophyte, the diameter of *F. culmorum* (Fu42) colonies averaged 39 mm after 4 days. However, when grown adjacent to 4-day-old colonies of *A. alternata, B. cinerea* or *C. herbarum*, the diameter of *F. culmorum* colonies was reduced to 24, 19 and 13mm, respectively. The diameter of *M. nivale* (M15M2) colonies averaged 20 mm after 4 days. However, when grown adjacent to 4-day old colonies of *A. alternata, B. cinerea* or *C. herbarum*, the diameter of *M. nivale* was reduced to 9, 5 and 6 mm, respectively (Figure 10). For all three saprophytic species, delaying the introduction of *F. culmorum* or *M. nivale* resulted in greater inhibition of the pathogen. A clear zone of inhibition was observed between *A. alternata* and both pathogens, measuring approximately 1-2mm.

Table 16. Effect of artificially inoculating ears of winter wheat (cv. Avalon) with *Alternaria alternata, Botrytis cinerea* or *Cladosporium herbarum* at GS 59 or GS 69 (Zadoks *et al.*, 1974) on the development of Fusarium ear blight (FEB) symptoms assessed at GS 75 after artificial inoculation of ears with *M.nivale* at GS 65.

ORGANISM AND TIME OF INOCULATION			% SPIKELETS INFECTED	
GS 59	GS 65	GS 69	GS 75	GS 80
A.alternata			0	0
B.cinerea			0	0
C.herbarum			0	0
	M.nivale		1.0	7.9
A.alternata	*M.nivale*		2.1	10.5
B.cinerea	*M.nivale*		1.5	7.0
C.herbarum	*M.nivale*		8.9	17.2
	M.nivale	*A.alternata*	3.4	5.5
	M.nivale	*B.cinerea*	4.8	12.7
	M.nivale	*C.herbarum*	10.1	16.9
		LSD (p=5%)	6.5	10.3
		40 df		

ANOVA F probabilities;
GS 75: treatment <0.001
SED (treatment) = 2.959, 8 df
GS 80: treatment <0.001
SED (treatment) = 4.551, 8 df

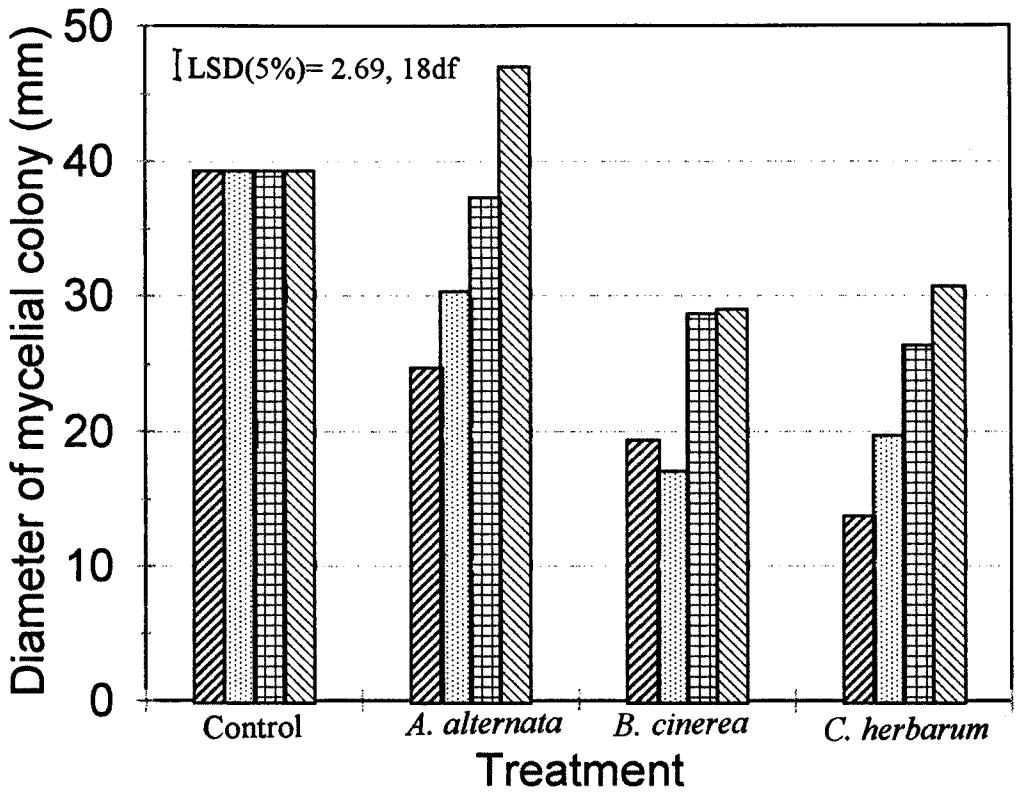


Figure 9. Mean diameter of *Fusarium culmorum* (Fu42) colonies after 4 days incubation at 20° when placed onto PDA bearing 4d (▨), 3d (▩), 2d (▦) or 1d (▧) old cultures of either *A. alternata*, *B. cinerea* or *C. herbarum*. The LSD bar represents the LSD for treatment/block interactions effects. Control treatment was *F. culmorum* inoculated opposite to *F. culmorum*.

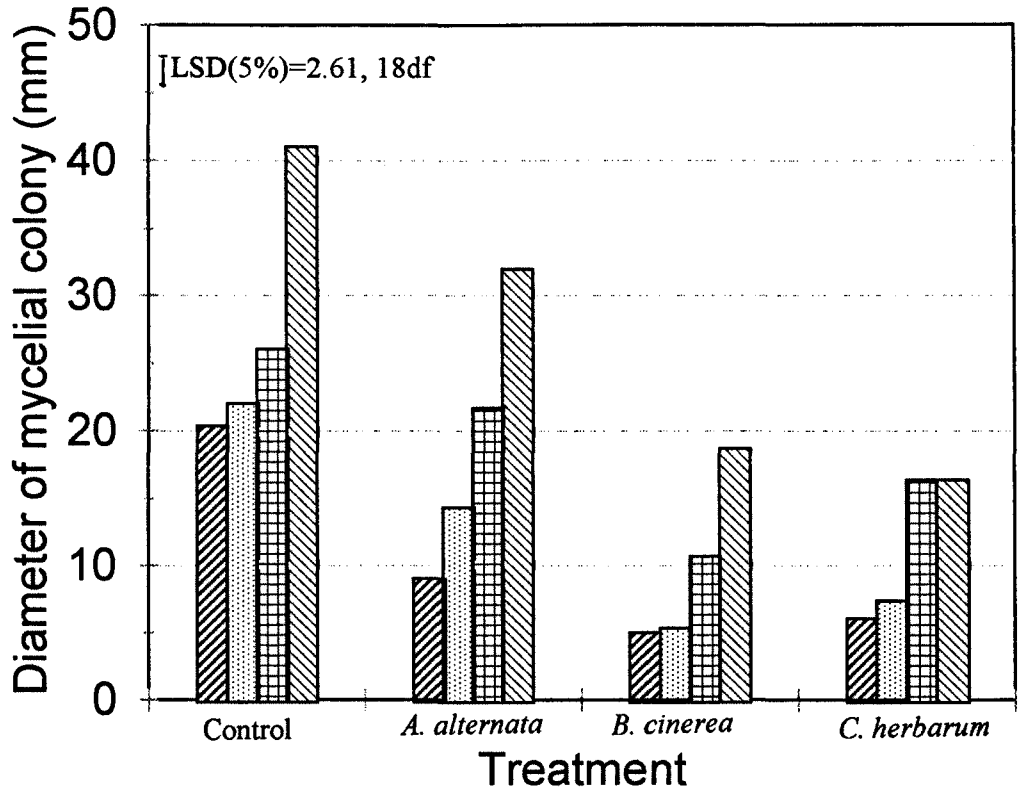


Figure 10. Mean diameter of *Microdochium nivale* (M15M2) colonies after 4 days incubation at 20°C when placed onto PDA which had 4d (▨), 3d (▩), 2d (▦)or 1d (▧) old colonies of either *A. alternata, B. cinerea* or *C. herbarum*.The LSD bar represents the LSD for treatment/block interactions effects. The control was *M. nivale* inoculated opposite *M. nivale*.

Determination of non-volatile antibiosis

In tests to determine whether antibiosis shown in dual culture resulted from the production of non-volatile antibiotics, a significant ($p<0.05$) reduction in mycelial growth occurred when *F. culmorum* or *M. nivale* was inoculated onto plates on which the saprophytes had been incubated prior to the pathogen. Mycelial colonies of the *F. culmorum* isolate Fu42 after 4 days were reduced from approximately 70 mm in control plates to 25, 11 and 20 mm for *A. alternata, B. cinerea* and *C. herbarum,* respectively, when the saprophytes were inoculated onto PDA 4 days prior to *F. culmorum* (Figure 11). Mycelial colonies of *M. nivale* isolate M15M2, after 4 days was reduced from approximately 55 mm to 42, 16 and 22 mm for *A. alternata, B. cinerea* and *C. herbarum,* respectively, when the saprophytes were inoculated onto PDA 2 days prior to *M.nivale* (Figure 12). There was self inhibition of *M. nivale* when inoculated onto plates on which *M. nivale* had been growing for 4 or 3 days. In general, the antibiotic effect of saprophytic microflora on the pathogens was reduced when the pre-inoculation period was reduced.

Determination of volatile antibiosis

In tests to determine whether antibiosis shown in dual culture resulted from the production of volatile antibiotics, the greatest reduction in growth was usually recorded for those isolates incubated above *C. herbarum* (Figure 13). For all three saprophytes, mycelial colonies of *F. culmorum* after 5 days measured 40 to 50mm compared to 80mm in control plates. Where *M. nivale* had been incubated above *A. alternata, B. cinerea* and *C. herbarum*, mycelial colonies of the pathogen after 5 days measured 10, 11.7 and 4.7, respectively, compared to 22 mm in control plates (Figure 14). Tests for volatile antibiosis showed that all saprophytes reduced the mycelial growth of both pathogens. This suggests that the reduction of mycelial growth of *F. culmorum* and *M. nivale* observed in dual culture experiments was due at least in part to

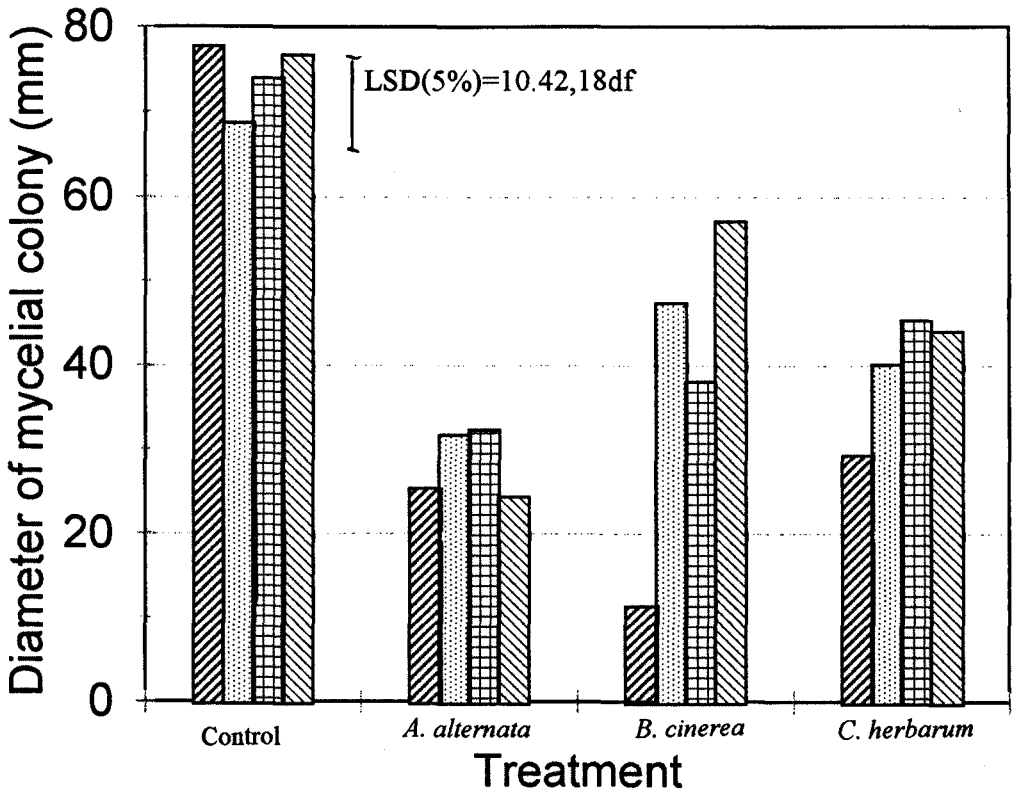


Figure 11. Mean diameter of *Fusarium culmorum* (Fu42) after 4 days incubation at 20°C when placed onto PDA which had 4d (▨), 3d (▩), 2d (▦)or 1d (▧) old colonies of either *A. alternata, B. cinerea* or *C. herbarum* removed prior to inoculation with *F. culmorum*.The LSD bar represents the LSD for treatment/block interactions effects. The control was *F. culmorum* inoculated onto PDA on which *F. culmorum* had been growing.

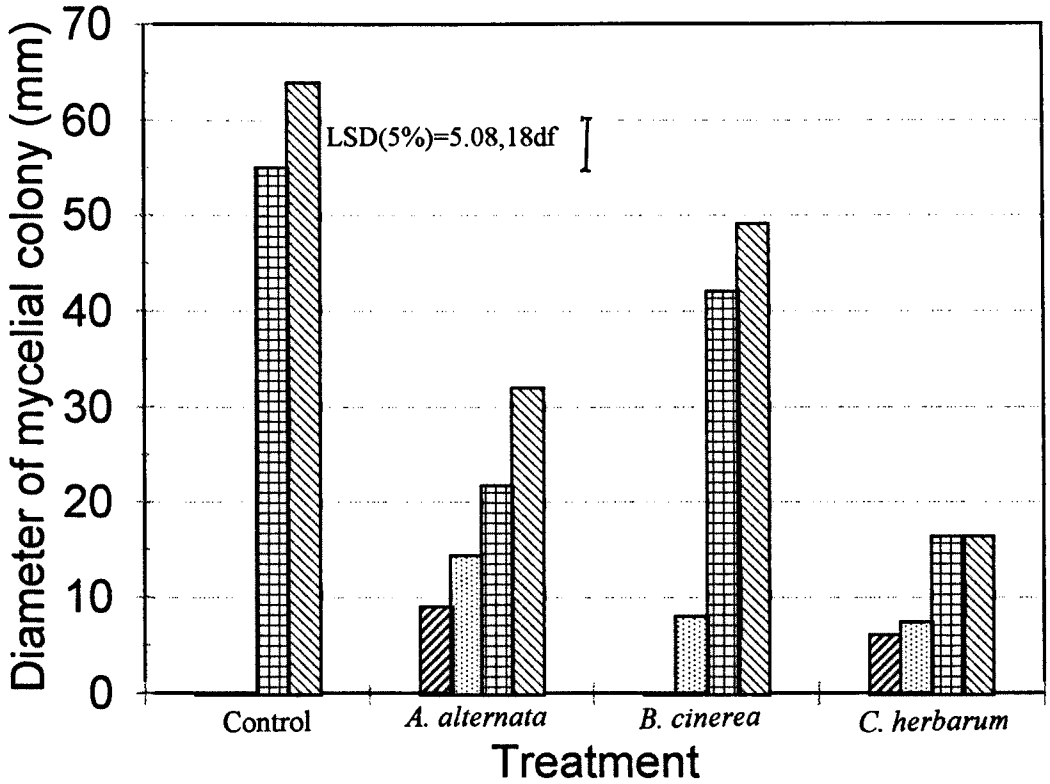


Figure 12. Mean diameter of *Microdochium nivale* (M15M2) colonies after 4 days incubation at 20°C when placed onto PDA which had 4d (▨), 3d (▩), 2d (▦) or 1d (▧) old colonies of either *A. alternata, B. cinerea* or *C. herbarum* removed prior to inoulation with M. Nivale.The LSD bar represents the LSD for treatment/block interactions effects. The control was *M. nivale* inoculated onto plates which had *M. nivale* removed.

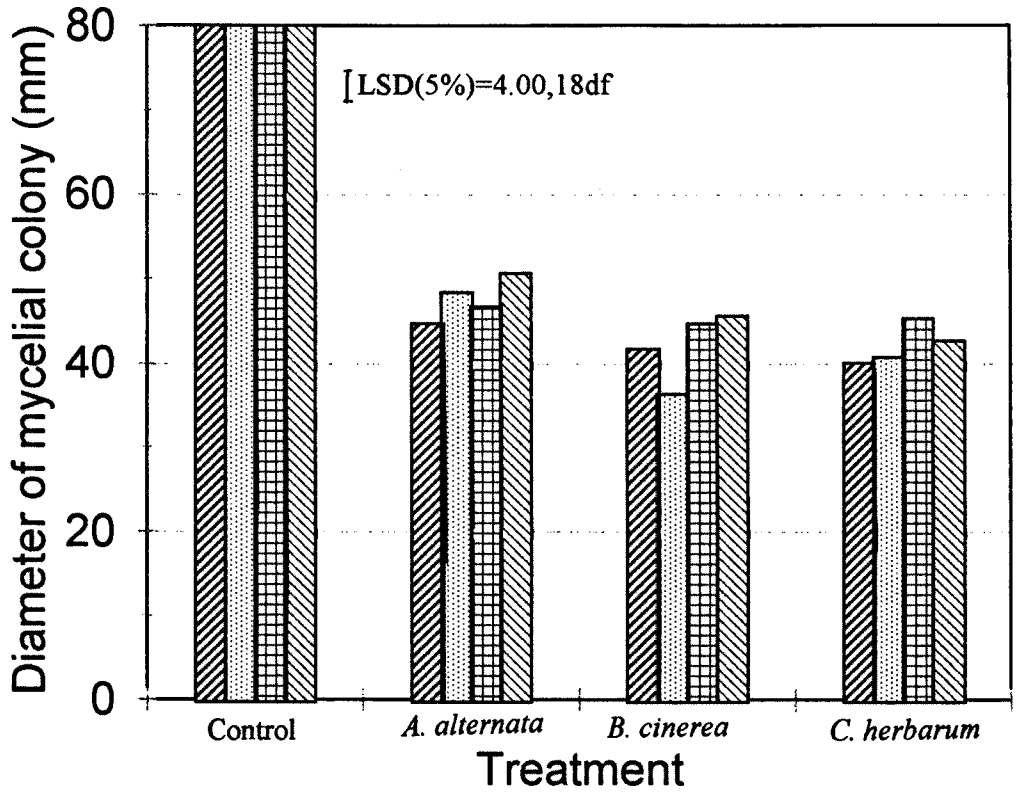


Figure 13. Mean diameter of *F. culmorum* (Fu42) colonies after 5 days incubation at 20°C directly above 4d (▨), 3d (▯), 2d (▦) or 1d (▧) old colonies of either *A. alternata, B. cinerea* or *C. herbarum*. The LSD bar represents the LSD for treatment/block interactions effects.

production of volatile substances by *A. alternata*, *B. cinerea* and *C. herbarum*.

Determination of antimycelial / antisporulant products

Analysis of variance showed no significant decrease ($p<0.05$) in mycelial growth of either pathogen due to the addition of cell free filtrates to the cultures for the isolates studied. For spore germination data, analysis of variance revealed that cell free filtrates of both *A. alternata* and *B. cinerea* significantly reduced germination of *F. culmorum* isolate Fu42 spores. However, when using *F. culmorum* isolate F200, a significant reduction in spore germination was only achieved by a cell free filtrate of *B. cinerea*. For the *Microdochium nivale* isolate M15M2, a significant reduction in spore germination was achieved only by a cell free filtrate of *B. cinerea* (Table 17). However, all cell free extracts reduced the spore germination of *M. nivale* isolate RFN_2I spores, with *B. cinerea* causing the most significant effect.

Table 17. Mean percentage spore germination of *Fusarium culmorum* and *Microdochium nivale* in the presence of cell free extracts of epiphytic microflora; *Alternaria alternata*, *Botrytis cinerea* and *Cladosporium herbarum* .

	Mean spore germination (%)			
Cell Free Filtrate	*F.culmorum* Fu42	*F.culmorum* F200	*M.nivale* M15M2	*M.nivale* RFN_2I
none	96.78	95.89	95.67	23.44
A.alternata	94.89	94.56	94.78	18.11
B.cinerea	82.00	90.56	24.67	6.89
C.herbarum	96.00	95.67	95.33	21.22

LSD ($p=0.05$) = 2.17

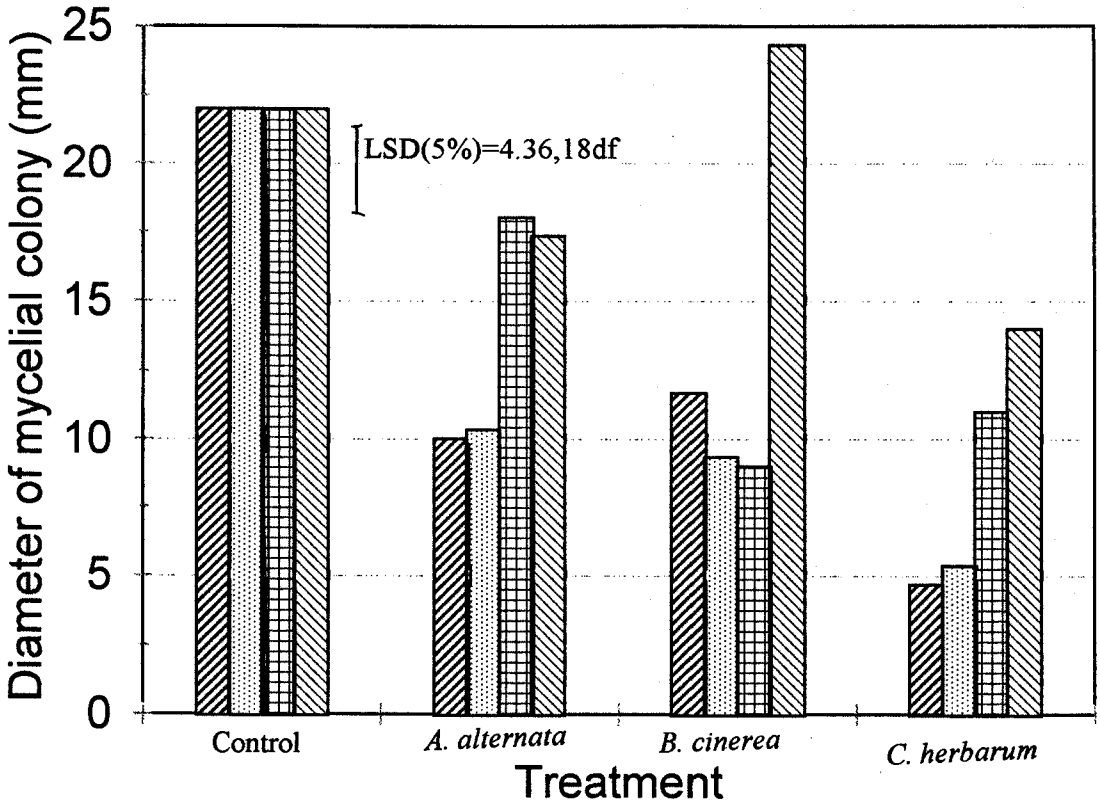


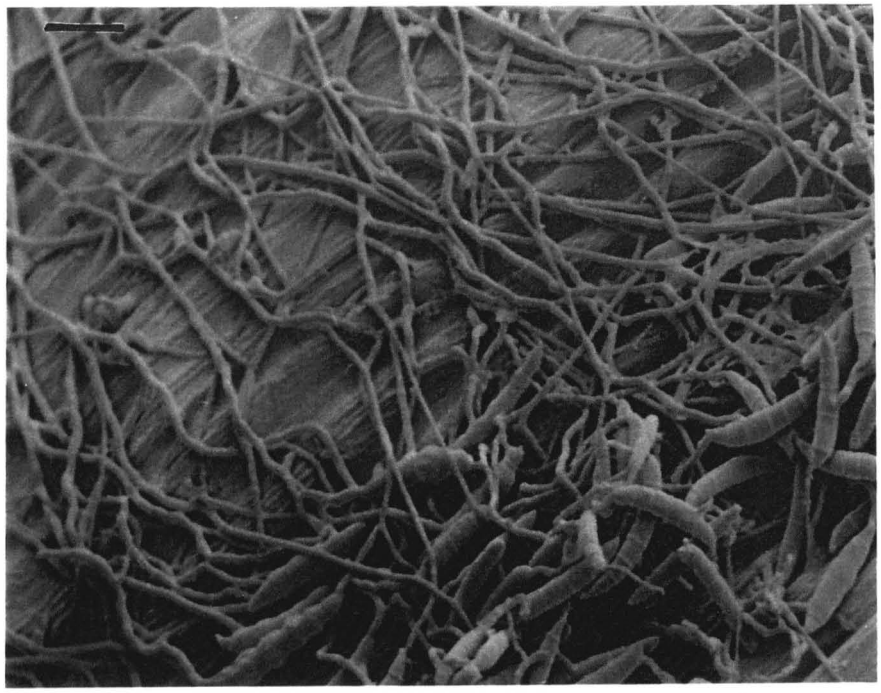
Figure 14. Mean diameter of *M. nivale* (M15M2) colonies after 5 days incubation at 20°C directly above 4d (▨), 3d (⬚), 2d (▦) or 1d (▧) old colonies of either *A. alternata, B. cinerea* or *C. herbarum*. The LSD bar represents the LSD for treatment/block interactions effects.

Electron Microscope Studies

Scanning electron microscopy studies of spikelets gave disappointing results. Due to the complex morphology of spikelets, it was difficult to observe spores and hyphal extension of pathogenic and saprophytic species. Grain was used as a model system to investigate the morphology of the interaction between the potential antagonists *A. alternata, B. cinerea* and *C. herbarum*, and *F. culmorum* as this yielded better results. In scanning electron microscope studies on grain, the potential antagonists *A. alternata, B. cinerea* and *C. herbarum* were rarely observed in close proximity to *F. culmorum* spores. Where spores and hyphae were observed together, there was no evidence of hyphal interactions or hyper-parasitism. On grain soaked in cell free filtrates of *A. alternata* and *B. cinerea*, there appeared to be a reduction in the germ tube length of *F. culmorum* spores in the crease of the grain, but not on the rest of the grain (Figures 15 to 18).

Figure 15. Spore germination and mycelial growth of *F. culmorum* on the surface of the main body of wheat grain which had previously been soaked in cell free filtrate of *B. cinerea*

Figure 16. Spore germination and mycelial growth of *F. culmorum* near the crease of wheat grain which had previously been soaked in cell free filtrate of *B. cinerea*, showing a reduction in germ tube length of *F. culmorum*



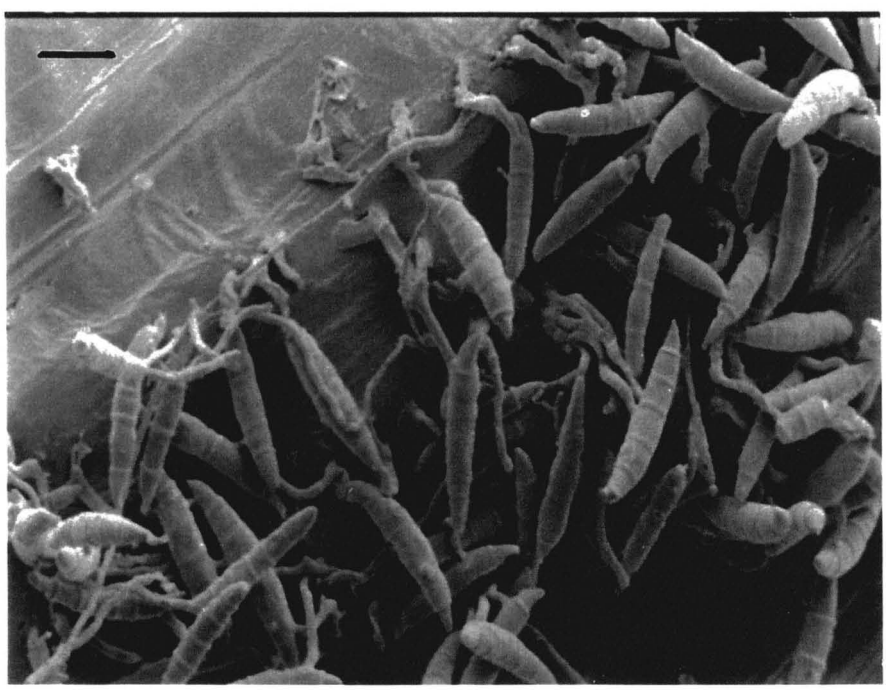


Figure 17. Spore germination and mycelial growth of *F. culmorum* on the surface of the main body of wheat grain which had previously been soaked in cell free filtrate of *A. alternata*

Figure 18. Spore germination and mycelial growth of *F. culmorum* near the crease of wheat grain which had previously been soaked in cell free filtrate of *A. alternata*, showing the presence of small, abnormal germ tube development of spores

Discussion

Glasshouse-grown plants inoculated with *A. alternata, B. cinerea* or *C. herbarum* prior to inoculation with *F. culmorum*, showed reduced severity of FEB. This suggests that prior colonization of spikelets by *A. alternata, B. cinerea* or *C. herbarum* reduces the potential of *F. culmorum* to colonise tissue and cause symptoms of disease. Where the saprophytic species were introduced to wheat ears following inoculation with *F. culmorum*, no significant reduction in disease was recorded. This may indicate that saprophytic species are unable to compete with *F. culmorum* once it has infected spikelets and become established on the ear. The absence of any significant reduction in FEB due to inoculation of ears with *A. alternata* or *B. cinerea* prior to *M. nivale*, suggests that these saprophytes were not antagonistic to *M. nivale in vivo*. This highlights the differences in behaviour between *Fusarium* species and *Microdochium nivale*. The considerable increase in the severity of FEB when *C. herbarum* was inoculated either prior to or after inoculation of *M. nivale*, may have been due to increased senescence of the tissue caused by *C. herbarum* (Jachmann and Fehrmann, 1989) or may have been associated with the production of substances stimulatory to the pathogen (Valadon and Lodge, 1970). Further studies are recommended to investigate whether *C. herbarum* does increase senescence and whether it produces substances stimulatory to other fungal colonisers.

Wainwright (1992) washed fungal spores from the ears of wheat and showed that spores of *Alternaria* spp., *B. cinerea* and *Cladosporium* spp. were present on ears of wheat at GS 59. These results indicate that saprophytic species colonise ears before flowering. The *in vitro* experiments showed that antibiosis occurred between saprophytes and pathogens, and suggests that the mode of action of this antagonism may be due to the production of both non-volatile and volatile antibiotics. Dennis and Webster (1971a) showed that many species of *Trichoderma*, including *Trichoderma piluliferum, T. polysporum* and *T. viride* produced non-

volatile antibiotics which inhibited mycelial growth of *Fomes annosus in vitro*. Volatile antibiotic production by *Trichoderma viride* was also shown to reduce mycelial growth of *Fusarium oxysporum in vitro* by up to 19 % (Dennis and Webster, 1971b). Antibiosis mediated by volatile production is effective within a closed environment, however, such antagonism is unlikely to cause significant effects in the field situation. Fokkema *et al.* (1975) investigated the buffering capacity of natural microflora of rye leaves to infection by *Cochliobolus sativus*. The density of saprophytes was determined from leaf washings over a three year period and related to the necrotic leaf area caused by *C. sativus* following artificial inoculation at anthesis. The higher densities of *Sporobolomyces roseus*, *Cryptococcus* spp, *Cladosporium* spp. and *Aureobasidium pullulans* were correlated with lower levels of infection by *C. sativus*. This suggested a "buffering effect" of phyllosphere fungi on leaves which may occur on ears.

Scanning electron microscopy showed that pathogenic and saprophytic species were rarely observed in close proximity. This may have been due to an inappropriate inoculation procedure. Alternatively, antibiotic activity by *A. alternata*, *B. cinerea* and *C. herbarum* may have caused inhibition of adhesion and germination of the conidia of *F. culmorum* in close proximity to the spores and hyphae of the saprophytic species. This might also imply that competition for nutrients and space are the key factors involved in the relationship between *F. culmorum*, *A. alternata*, *B. cinerea* and *C. herbarum*, and this itself is a type of antagonism (Dik, 1992). Unfortunately, no spore counts were made to determine quantitatively whether adhesion and germination had occurred. This could be an area for more extensive study. Scanning electron microscopy of grain which had been soaked in the cell free filtrates of *A. alternata*, *B. cinerea* or *C. herbarum* prior to inoculation, showed there was a decrease in the length of germ tubes of *F. culmorum* spores colonising near the crease, however, such results were not quantified. This may have been due to either a decrease in the germination of conidia in the crease or the

crease had absorbed more of the cell free filtrate which reduced spore germination and germ tube extension. The pH of the *B. cinerea* extract was 4.2 compared to approximately pH 7 for all the other extracts, hence this may to some extent explain the inhibition of spore germination of *F. culmorum* on grain soaked in the cell free filtrate of *B. cinerea*. However, this does not explain the results for *A. alternata*. The antibiotic activity of cell free filtrates should be investigated further using light microscopy, to provide quantitative measurements.

There are limitations to the use of *in vitro* studies to determine the relationships between fungal pathogen and saprophytic microflora. Deacon (1991) reviewed the significance of ecology in the development of biocontrol agents against soil-borne plant pathogens and criticized the selection of biocontrol agents on the basis of their performance in *in vitro* experiments. He stated that, often, biocontrol agents selected in this way are unsuitable for the environment in which the pathogen grows. The environment *in vitro* is very remote from the environment in which the organism would be placed. For example, Reinecke and Fokkema (1981), whilst evaluating methods for screening fungi for antagonism to *P. herpotrichoides* argued that Petri-dishes were inadequate since *Microdochium bolleyi* was shown to be only weakly antagonistic *in vivo* whereas Domsch and Gams (1968) suggested that it was strongly antagonistic as a result of *in vitro* tests. Hence, there are limitations of *in vitro* experiments which should be considered when interpreting the apparently antagonistic relationships between pathogens and saprophytes.

Bansal *et al.* (1988) used a combination of *in vitro* and glasshouse experiments to screen for biological control of *Helminthosporium* blight of wheat by phylloplane microflora. They found that among a range of 11 fungi and bacteria, some were antagonistic to *Helminthosporium* sp. For example, *B. cinerea* caused a 48 % reduction in mycelial growth of the pathogen and

caused a zone of inhibition of 15mm, indicating that antibiotic production by *B. cinerea* may be partly responsible for the inhibition of mycelial growth. In the glasshouse there was a 47.5 % reduction in the incidence of leaf spot on the flag leaves attributed to the inoculation of *B. cinerea*, hence, the results of *in vitro* and glasshouse experiments were comparable. These results compare favourably with those recorded for suppression of *F. culmorum* during *in vitro* and glasshouse experiments reported here, although the control of *Helminthosporium spp.* reported by Bansal *et al.* (1988) was greater than that for *F. culmorum*. *Botrytis cinerea* showed antagonistic activity against *Helminthosporium* spp. and *F. culmorum* in both glasshouse and *in vitro* experiments, but to different degrees, showing that the two pathogens vary in sensitivity to *B. cinerea.*

This work has shown the potential of common saprophytic flora of wheat ears to suppress FEB, although there may be another complicating factor. It is possible that the inoculation of ears prior to the pathogens caused the manufacture of defence related compounds in the tissue, such as chitinase, fungal cell wall degrading enzymes, enrichment of plant cell wall proteins such as hydroxyproline rich glycoproteins and non-protein components lignin and cell wall phenolics (Bolwell *et al.*, 1985). Hence, the reduction in FEB may have been due to 'triggered' plant defence system rather than an antagonism between pathogens and saprophytes *per se*. For example, the enrichment of cell walls with hydroxyproline was demonstrated by Thorpe and Hall (1984) who inoculated wheat leaves with *B. cinerea*. Increased peroxidase activity was demonstrated and the peroxidase was shown to be localised around the margins of infected wounds where lignification occurred. There is little evidence to determine whether microflora such as *A. alternata, B. cinerea* and *C. herbarum* trigger a defence response in plants which would explain the reduction of disease. This would be an interesting area for research but is beyond the scope of this project.

CHAPTER 6

The Effect of Selected Fungicides on the Colonisation of Ears by Saprophytic Microflora of Winter Wheat

Introduction

The aim of these studies was to determine the effects of fungicides on ear microflora using *in vitro* and glasshouse experiments. In addition, an experiment was set-up *in vitro* to determine potential antagonism between *A. alternata, B. cinerea* or *C. herbarum* and *F. culmorum* or *M. nivale* in the presence of a range of fungicides.

Materials and Methods

The effect of fungicides on the growth of four fungal species *in vitro* was determined by inoculating them onto PDA amended with a range of concentrations of 8 fungicides *in vitro.* For each fungal isolate, 5mm plugs were placed centrally onto separate PDA plates amended with fungicides (Table 2, page 36) at concentrations of 0.05, 0.25, 1.25 and 2.00µg a.i ml^{-1} of agar. For each fungicide and for each concentration, five replicate plates were inoculated. The inoculation of PDA plates with no fungicide provided control treatments. After inoculation, all plates were sealed with parafilm and incubated in the dark for 5 days at 20°C +/- 2°C. After incubation, fungal colony diameters were recorded daily.

To determine the effect of fungicide application on the microflora of ears under glasshouse conditions, plants were inoculated with either *Alternaria alternata, Botrytis cinerea* or *Cladosporium herbarum* at a rate of 200,000 spores per ml at mid-anthesis. The ears were individually covered in self sealing clear plastic bags for 48 hours to induce infection and colonisation. One week following inoculation, the fungicides prochloraz, tebuconazole, chlorothalonil or pyrimethanil were applied at field rate using a precision pot sprayer (Chapter 2, page 35). Uninoculated ears treated with fungicides and uninoculated untreated ears provided control treatments. Three days following fungicide application, all ears were harvested and the colonisation by each fungal species determined.

The sampling procedure used was in accordance with that used by Wainwright *et al.* (1992).Ten ears were taken for each treatment. Five were placed in a 100ml glass jar and five in a second jar, each containing 50 ml of distilled water added to each plus 2 drops of Tween 80 wetting agent (polyoxyethylene (20) sorbitan mono-oleate, BDH Limited, Poole, England). The jars were then agitated by hand on rotation for 1 hour and the resulting spore suspension was decanted and stored at 4°C. Aliquots of 0.1 ml were spread evenly over PDA or PDA supplemented with streptomycin sulphate (100 μ g ml^{-1}), neomycin sulphate (50 μg ml^{-1}) and chloramphenicol (50 μg ml^{-1}). Five replicate plates were set-up for each treatment on each medium type. Plates were incubated at 20°C +/- 2°C for 48 hours and the colony forming units per cm^2 were counted. After a further 3 days, the fungal colonies were classified as *A. alternata, B. cinerea* or *C. herbarum*.

The effect of fungicides on the relationship between saprophytic microflora and the pathogens *F. culmorum* and *M. nivale* was determined *in vitro* using a dual culture procedure. Isolates of *F. culmorum* or *M. nivale* were inoculated opposite ear microflora on artificial media amended with fungicides *in vitro*. Isolates of *A. alternata, B. cinerea* and *C. herbarum* were grown in dual culture with *F. culmorum* (Fu42) or *M. nivale* (M15M2), based on the method described by Dennis and Webster (1971a). For each saprophytic species, a 5mm plug was taken from an actively growing colony and placed on a plate of PDA amended with 0.25, 1.25 or 2.00 μg ml^{-1} of either prochloraz, tebuconazole, chlorothalonil or pyrimethanil, 4 days prior to the introduction of a plug of the pathogen. Control plates contained no fungicide. Four replicate plates were inoculated for each treatment. The saprophyte and pathogen plugs were placed 3cm apart. All plates were incubated at 20°C for 7 days. After incubation, the diameter of all pathogen colonies were measured.

Results

In vitro studies on the effect of fungicides on the growth of *A. alternata, B. cinerea* and *C. herbarum* showed that each fungicide demonstrated a differential activity against the fungal species tested. For example, pyrimethanil reduced mycelial growth of *B. cinerea* by up to 93 % yet failed to reduce the mycelial growth of *A. alternata* or *C. herbarum* by more than 28 % (Table 18). Benomyl reduced mycelial growth of *B. cinerea* by up to 92 %, but failed to reduce mycelial growth of *A. alternata* by more than 17 % and *C. herbarum* by more than 63 %. Flutriafol reduced the mycelial growth of *B. cinerea* by 26 % but was more effective against *A. alternata* and *C. herbarum* which were reduced by 72 and 61 % respectively. Tebuconazole was consistently effective at reducing the mycelial growth of all three species by over 69 %.

In the glasshouse, plants inoculated with either *A. alternata, B. cinerea* or *C. herbarum* did not show any symptoms of disease, however, the saprophytes were re-isolated from the ears five days after inoculation. The colony forming units (cfu) re-isolated after 48 hours were shown to be mainly pink and white yeasts (data not shown). The number of colony forming units of *A.alternata, B.cinerea* and *C.herbarum* recovered from ears treated with fungicides and inoculated with those species, are shown in Figures 19, 20 and 21 respectively.

Application of prochloraz to plants inoculated with *A. alternata* led to a significant ($p<0.05$) increase in the re-isolation of *A. alternata* from 2.16 colonies per plate of PDA for the control to 6.4 colonies per plate, whereas applications of either tebuconazole or chlorothalonil, led to a significant decrease in the re-isolation of this saprophyte (Figure 19). There was no significant effect on the re-isolation of *A. alternata* from ears treated with pyrimethanil when washings were plated onto PDA, but a significant increase in re-isolation of this species when plated onto antibiotic amended PDA. For the re-isolation of *B. cinerea*, from plants inoculated with this

saprophyte, prochloraz and chlorothalonil led to a significant decrease in the re-isolation of this saprophyte, for example from 4.4 for control plates to 0.42 and 2.8 colonies per plate of antibiotic amended PDA respectively, but tebuconazole led to a significant increase in its re-isolation on PDA from 8.2 to 13.4 (Figure 20). For ears inoculated with *C. herbarum*, there was a significant reduction in the re-isolation of *C. herbarum* following application of any of prochloraz, tebuconazole, chlorothalonil or pyrimethanil, irrespective of the re-isolation media (Figure 21).

Dual culture experiments carried out to investigate the effect of saprophytic microflora on the colony diameter of *F. culmorum* and *M. nivale* colonies on fungicide amended media showed the complexity of the relationships between the pathogens and saprophytic microflora species. When inoculated in the absence of fungicides, *Botrytis cinerea* led to a significant reduction in the diameter of *F. culmorum* colonies from 25.8 to 4.8 mm. The fungicides prochloraz and tebuconazole led to a significant reduction in the diameter of *F. culmorum*. The greatest reduction occurred at 2 $\mu g\ ml^{-1}$, up to complete control of the pathogen. The fungicides chlorothalonil and pyrimethanil failed to cause a significant reduction in colony diameter, even at the highest concentration. In the presence of *A. alternata*, *B. cinerea* and *C. herbarum* on prochloraz-amended media, there was a greater reduction in the diameter of *F. culmoum* with increasing concentration of prochloraz, irrespective of the presence of the microflora species. This illustrates the efficacy of prochloraz in controlling *F. culmorum in vitro*. The greatest reduction in *F. culmorum* diameter occurred in the presence of *B. cinerea* on prochloraz-amended media; a reduction from 25.8 mm for the control to 7.5, 5.8 and mm at 0.25, 1.25 and 2 $\mu g\ ml^{-1}$ prochloraz in the presence of *B. cinerea*. Similarly for tebuconazole, there was greater reduction in the colony diameter of *F. culmorum* at the highest concentrations of tebuconazole, irrespective of the microflora species present.

Table 18. Mean % reduction in mycelial colony diameter of three fungal species when grown on PDA amended with a range of fungicides and concentrations (numbers in parentheses are standard errors).

		Mean reduction in colony diameter (%)		
Fungicide	Conc (µg ml^{-1})	*A. alternata*	*B. cinerea*	*C. herbarum*
benomyl	0.05	10.9 (0.67)	53.91 (0.26)	15.27 (1.21)
	0.25	13.4 (0.49)	80.47 (1.09)	9.92 (0.52)
	1.25	14.6 (0.58)	92.19 (0.42)	23.66 (0.67)
	2.00	17.1 (0.32)	92.19 (0.92)	62.60 (0.26)
chlorothalonil	0.05	0 (0.64)	14.06 (0.92)	11.31 (0.26)
	0.25	3.5 (0.74)	18.75 (0.49)	19.05 (1.09)
	1.25	4.7 (0.67)	80.47 (0)	20.83 (0.42)
	2.00	8.1 (0.52)	97.66 (0.52)	29.76 (0.92)
fluquinconazole	0.05	7.7 (0.52)	12.87 (0.67)	22.14 (0)
	0.25	62.2 (0.26)	49.50 (0.26)	38.93 (0)
	1.25	70.7 (0.32)	77.23 (0.32)	53.44 (0.47)
	2.00	61.4 (0.64)	91.09 (0.76)	58.78 (0.26)
flusilazole	0.05	20.9 (0.32)	9.84 (1.19)	23.81 (0.63)
	0.25	52.3 (0.26)	13.11 (0.60)	63.10 (0.31)
	1.25	74.4 (0.32)	34.43 (0)	53.57 (0.31)
	2.00	94.2 (0)	52.46 (1.08)	58.33 (0.31)
flutriafol	0.05	4.7 (0.52)	19.67 (0.60)	25.00 (0.31)
	0.25	18.6 (0.42)	59.02 (1.40)	36.31 (0.37)
	1.25	60.5 (0.49)	14.75 (0.89)	57.74 (0.37)
	2.00	72.1 (0.26)	26.23 (1.08)	60.71 (0)

Table 18. (contd)

		Mean reduction in colony diameter (%)		
Fungicide	Conc (µg ml^{-1})	*A.alternata*	*B.cinerea*	*C.herbarum*
prochloraz	0.05	76.5 (3.00)	67.3 (0.49)	78.0 (0.26)
	0.25	82.5 (5.71)	80.8 (0)	94.7 (0.52)
	1.25	91.0 (3.88)	89.4 (0.26)	100 (0.32)
	2.00	93.6 (3.35)	96.2 (0.26)	100 (0.52)
pyrimethanil	0.05	4.5 (2.07)	75.6 (0)	16.8 (0.26)
	0.25	1.9 (1.52)	84.2 (0.32)	5.3 (0.26)
	1.25	6.5 (1.39)	90.2 (0.32)	24.4 (0.32)
	2.00	9.8 (1.36)	92.7 (0.89)	27.8 (0)
tebuconazole	0.05	12.8 (0)	25.8 (0.49)	41.1 (0.49)
	0.25	38.4 (0.52)	57.5 (0.82)	56.6 (0.26)
	1.25	72.1 (0.49)	63.5 (1.09)	78.6 (0.26)
	2.00	81.4 (0.76)	69.6 (0.71)	75.6 (0.32)

SED (fungicide) = 0.645, 25 df

SED (concentration) = 0.51, 40 df

SED (fungicide*concentration) = 1.442, 5 df

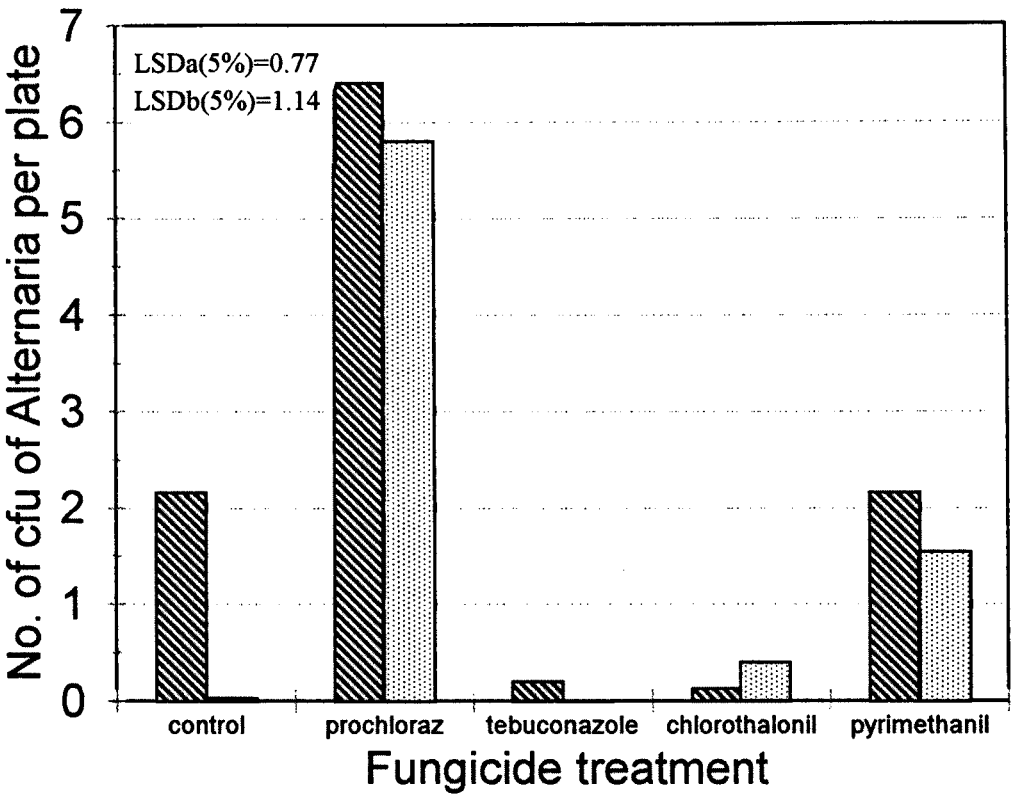


Figure 19. The effect of applying prochloraz, tebuconazole, chlorothalonil or pyrimethanil to ears of winter wheat (cv Avalon), on the re-isolation of *Alternaria alternata* from ears which had been inoculated with the saprophyte 48 hours prior to fungicide application The mean number of colonies per plate of PDA (▨) or antibiotic amended PDA (▨) was recorded after 5 days incubation. LSDa refers to the LSD on PDA and LSDb refers to the LSD on antibiotic amended PDA.

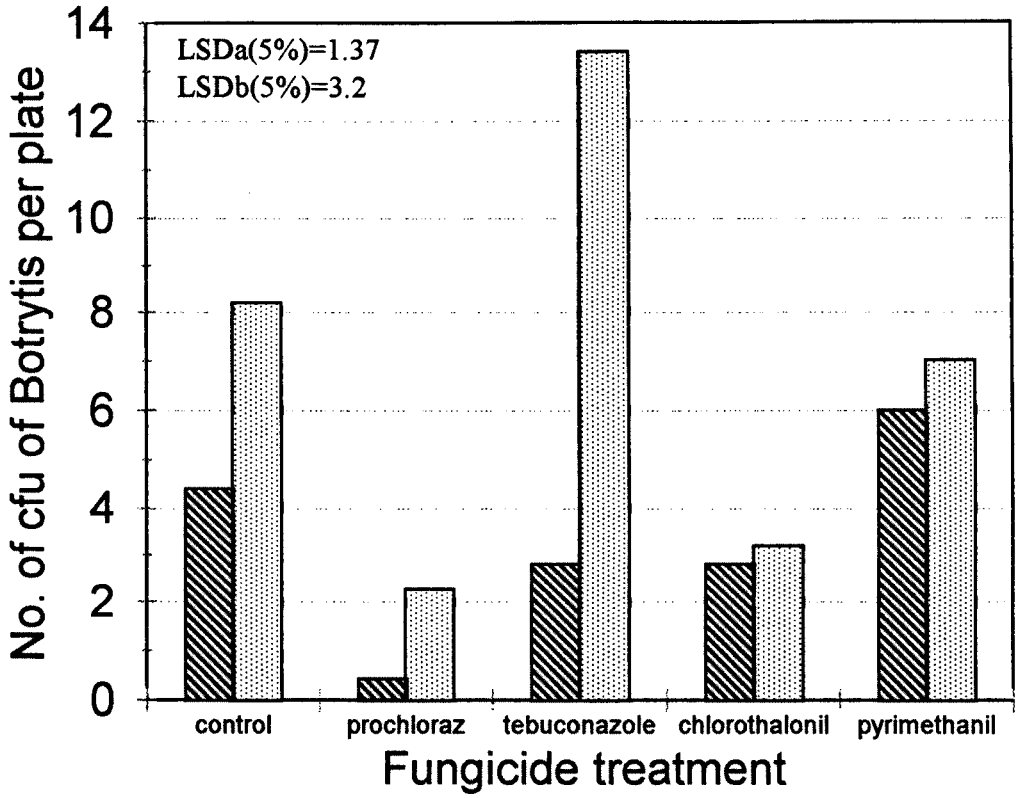


Figure 20. The effect of applying either prochloraz, tebuconazole, chlorothalonil or pyrimethanil to ears of winter wheat (cv Avalon) on the re-isolation of *Botrytis cinerea* from ears which had been inoculated with the saprophyte 48 hours prior to fungicide application. The mean number of colonies per plate of PDA (▨) or antibiotic amended PDA (□) was recorded after 5days incubation. LSDa refers to the LSD on PDA and LSDb refers to the LSD on antibiotic amended PDA.

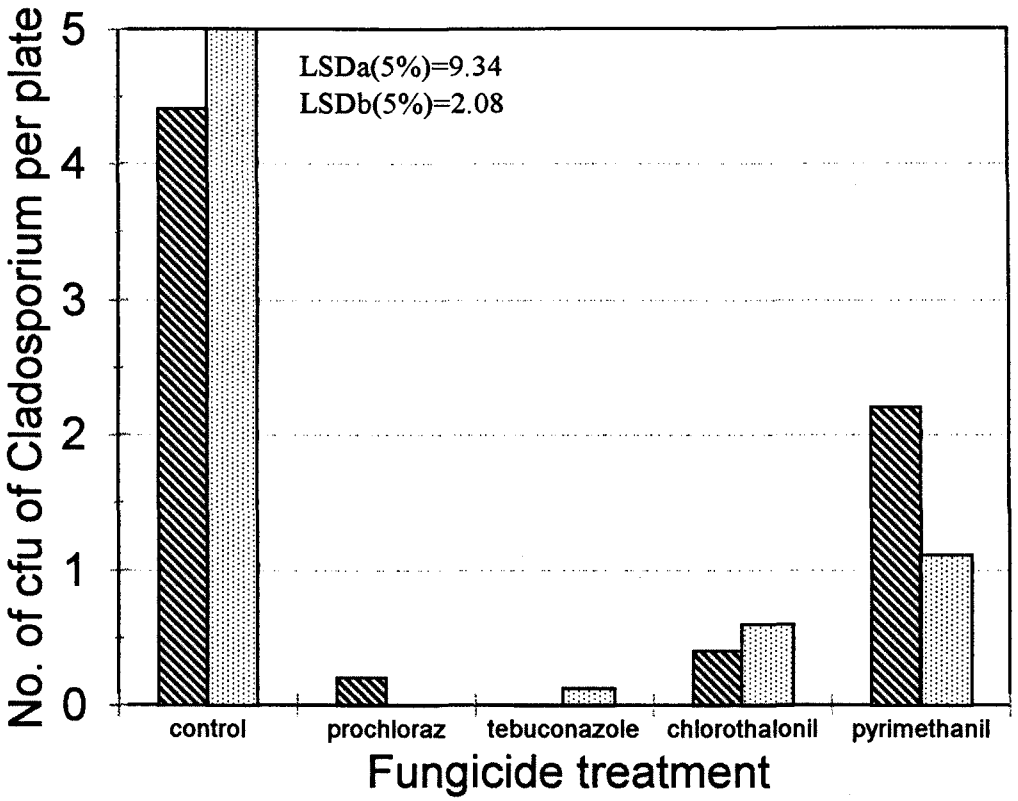


Figure 21. The effect of applying either prochloraz, tebuconazole, chlorothalonil or pyrimethanil to ears of winter wheat (cv Avalon) on the re-isolation of *Cladosporium herbarum* from ears which had been inoculated with the saprophyte 48 hours prior to fungicide application. The mean number of colonies per plate of PDA (▨) or antibiotic amended PDA (▨) was recorded after 5 days incubation. LSDa refers to the LSD on PDA and LSDb refers to the LSD on antibiotic amended PDA.

Table 19. Mean Diameter of mycelium (mm) of *F. culmorum* colonies on PDA amended with four fungicides and bearing 4 day old colonies of either *A. alternata, B. cinerea* or *C. herbarum* after 7 days at 20 °C (number in parentheses are standard errors).

		Colony Diameter (mm)			
Fungicide	Concentration ($\mu g\ ml^{-1}$)	*F.culmorum* control	*A.alternata*	*B.cinerea*	*C.herbarum*
none		25.8 (0.96)	28 (1.83)	4.8 (0.50)	26.5 (11.39)
prochloraz	0.25	14 (1.83)	15.3 (0.96)	7.5 (1.73)	21.5 (1.91)
	1.25	9 (1.63)	7.3 (4.92)	5.8 (3.77)	10.8 (6.18)
	2.00	0 (0)	0 (0)	0 (0)	0 (0)
tebuconazole	0.25	15.5 (1.29)	18.5(2.08)	8.3 (2.75)	32.5 (3.11)
	1.25	6.5 (1.73)	7 (1.53)	2.8 (0.50)	6.8 (0.96)
	2.00	2 (0)	2 (1.41)	1.8 (0.50)	1.8 (0.5)
chlorothalonil	0.25	26 (0.82)	28.5 (1.73)	9.3 (13.84)	26.3 (0.96)
	1.25	27.3 (2.06)	21 (4.76)	7.5 (5.00)	27 (4.24)
	2.00	29 (3.27)	21.5 (2.65)	25.8 (4.65)	23 (7.62)
pyrimethanil	0.25	25 (0)	43 (4.24)	40.8 (9.91)	28.5 (1.73)
	1.25	40.3 (4.11)	49 (0.82)	42.5 (4.73)	29.5 (1.73)
	2.00	28.5 (2.38)	43.5 (0.58)	44.5 (3.11)	34 (2.45)

SED (microflora species) = 0.802, 52 df

SED (fungicide) = 1.294, 20 df

SED (microflora*species) =2.893, 4 df

F. culmorum showed tolerance of chlorothalonil and pyrimethanil in the absence of saprophytic microflora species and no significant reduction in colony diameter was recorded. At the lower concentrations of chlorothalonil in the presence of *B. cinerea* there was a significant reduction in the diameter of *F. culmorum*, suggesting that the *B. cinerea* was having an inhibitory effect on *F. culmorum*, because it was not affected by the chlorothalonil at concentrations of 0.25 and 1.25 $\mu g\ ml^{-1}$. On media amended with pyrimethanil, there was a significant increase in the diameter of *F. culmorum* in the presence of *A. alternata* and *B. cinerea*, and to a lesser extent *C. herbarum* up to 49, 44.5 and 34 mm, respectively.

In the experiments with *Microdochium nivale*, prochloraz at all concentrations, tebuconazole at 2 $\mu g\ ml^{-1}$ and pyrimethanil at 2 $\mu g\ ml^{-1}$ led to a significant reduction in the diameter of *M. nivale* in the absence of the saprophytic species. In addition, the presence of existing colonies of *B. cinerea* and *C. herbarum* led to a significant reduction in *M. nivale* colony diameter in the absence of fungicides. The addition of prochloraz led to the most significant reduction in colony diameter, irrespective of the presence of *A. alternata, B. cinerea* or *C. herbarum*. The presence of saprophytic microflora on media amended with pyrimethanil led to a reduction in the colony diameter of *M. nivale*, the most significant reduction from 28.3 mm for the control to between 15 and 19.8 mm at concentrations of 2 $\mu g\ ml^{-1}$. The presence of chlorothalonil and tebuconazole on media inoculated with *C. herbarum* led to an increase to up to 37.8 and 42.8 mm respectively.

Table 20. Mean diameter of mycelium (mm) of *M. nivale* colonies on PDA amended with four fungicides and bearing 4 day old colonies of either *A. alternata, B. cinerea* or *C. herbarum* after 7 days incubation at 20°C (number in parentheses are standard errors).

		Colony Diameter (mm)			
Fungicide	Concentration ($\mu g\ ml^{-1}$)	*M.nivale* control	*A.alternata*	*B.cinerea*	*C.herbarum*
none		28.3 (2.06)	26.8 (1.50)	2.8 (2.06)	1.3 (2.5)
prochloraz	0.25	0 (0)	26 (1.15)	0.3 (0.50)	11.5 (13.50)
	1.25	0 (0)	0 (0)	6.5 (6.24)	0 (0)
	2.00	0 (0)	1.3 (2.5)	0 (0)	1.3 (2.5)
tebuconazole	0.25	23.3 (1.5)	33.3 (2.06)	25.3 (0.50)	39.3 (0.5)
	1.25	15.0 (3.56)	30.5 (2.38)	30.3 (9.50)	37.5 (4.36)
	2.00	18.8 (1.71)	29 (2.16)	31.3 (2.36)	42.8 (2.63)
chlorothalonil	0.25	24.8 (0.50)	25.8 (3.69)	19.5 (2.38)	24.8 (0.5)
	1.25	21 (0.96)	26.8 (1.71)	20.5 (4.12)	34 (6.22)
	2.00	26.8 (2.36)	27.3 (1.5)	27.3 (2.63)	37.8 (2.06)
pyrimethanil	0.25	24.5 (0.58)	36 (6.38)	40.3 (3.30)	21.3 (3.40)
	1.25	21.3 (0.50)	39.8 (3.77)	40 (4.97)	20 (2.16)
	2.00	15.3 (1.50)	15 (2.16)	19.8 (4.57)	18.5 (1.00)

SED (microflora species) = 0.865, 52 df

SED (fungicide) = 1.395, 20 df

SED (microflora*species) = 3.117, 4 df

Discussion

It is evident from the *in vitro* mycelial growth experiments, that each of the three fungal species reacted differently to the fungicides tested. For *A. alternata, B. cinerea* and *C. herbarum*, prochloraz was inhibitory (>90 % at 2 $\mu g\ ml^{-1}$) and for *A. alternata*, flusilazole also reduced growth by over 90 %. In addition, growth of *B. cinerea* was reduced by over 90 % on media amended with benomyl, chlorothalonil, fluquinconazole and pyrimethanil. These results support those recorded by Smiley *et al.*, (1993) who showed that *C. herbarum* was highly sensitive to prochloraz, showing an ED_{100} value of less than $1\mu g.ml^{-1}$, of moderate sensitivity to chlorothalonil (ED_{50} $<1\mu g.ml^{-1}$, ED_{100} $>10\mu g.ml^{-1}$) and moderately tolerant of benomyl (ED_{50} $>1\mu g.ml^{-1}$, ED_{100} $>10\mu g.ml^{-1}$) . They also found that *Alternaria* was moderately tolerant of prochloraz and tolerant of benomyl and chlorothalonil. In their study, *Botrytis cinerea* was less affected by prochloraz and tebuconazole but more sensitive to benomyl and chlorothalonil *in vitro*. A similar study by Fokkema and de Nooij (1981) was undertaken to study the effect of fungicides on *Sporobolomyces roseus, Cryptococcus laurentii* var. *flavescens, Aureobasidium pullulans* and *Cladosporium cladosporioides*. All species varied in their sensitivity to the fungicides tested *in vitro* using fungicide amended media. The fungicides mancozeb (0.48 g l^{-1}), maneb (0.48 g l^{-1}), benomyl (0.05 g l^{-1}), carbendazim (0.05 g l^{-1}) and thiophanate-methyl (0.14 g l^{-1}) completely inhibited the mycelial growth of *C. cladosporioides*. Captafol (0.1 g l^{-1}), captan (0.12 g l^{-1}) and prochloraz (0.08 g l^{-1}) inhibited colony growth by 40 % compared with the untreated control. Thiram (0.08 g l^{-1}), tridemorph (0.11 g l^{-1}), triadimefon (0.03 g l^{-1}) and triforine (0.04 g l^{-1}) inhibited growth by 40-60 % of the control. Ethirimol (0.06 g l^{-1}), oxycarboxin (0.08 g l^{-1}) and sulphur (0.8 g l^{-1}) reduced the mycelial growth by greater than 60 % of the control. Although none of the fungal species used by Fokkema and de Nooij (1981) were used in the study presented here, there is clear evidence for inhibition of a wide range of saprophytic micro-organisms by a range of fungicides.

In the glasshouse, prochloraz application caused a significant increase in the re-isolation of *A. alternata*, although tebuconazole and chlorothalonil led to a decrease in its re-isolation from ears inoculated with *A. alternata*. The decrease in re-isolation of *A. alternata* following application of tebuconazole and chlorothalonil agree with the sensitivity of this species to the fungicides *in vitro*, however, *A. alternata* was also sensitive to prochloraz *in vitro*. For example, at 2 μl ml^{-1} prochloraz, there was a reduction in the mean mycelium diameter of 93.60 % which compares with 81.40 % with tebuconazole at this concentration. The reason for the poor activity of prochloraz against *A.alternata in vivo* is unknown, but may have been due to inappropriate time of application with respect to the lifecycle of *A. alternata*. Applications of prochloraz and chlorothalonil to ears inoculated with *B. cinerea* led to a significant decrease in the re-isolation of this species. This agrees with the sensitivity of *B. cinerea* to these two fungicides *in vitro*, however, on the basis of the experiments using fungicide amended media, it would be expected that re-isolation of *B. cinerea* from ears treated with tebuconazole would also be decreased. For *C. herbarum*-inoculated ears, all fungicides led to a decrease in the re-isolation of this species. It is possible that *C. herbarum* was more sensitive to the four fungicides than *B.cinerea* or *A. alternata*, although this disagrees with the results of the *in vitro* experiment which showed that only prochloraz and tebuconazole reduced the mycelial growth by over 70 % at 2 μg ml^{-1}.

It is important to note that although significant differences between treatments were apparent, only very low numbers of fungi were re-isolated from the ears. This may have been due to the re-isolation procedure or the time period before which the fungi were re-isolated or poor spore adhesion. For example, Bainbridge and Dickinson (1972) favoured the incubation of leaves on moist filter paper for the re-isolation of *C. herbarum*, tapwater agar for the re-isolation of *A. alternata* and PDA for the re-isolation of *B. cinerea*. They also advocated the use of antibiotics

alternata and PDA for the re-isolation of *B. cinerea*. They also advocated the use of antibiotics in media to reduce competition by bacterial contaminants on agar plates. Had the ears been removed and re-isolation occurred after a longer time interval, greater numbers of fungi may have been recovered to make the results more meaningful.

Comparable studies of the effect of fungicides on saprophytic microflora were made by Jenkyn and Prew (1973). Applications of benomyl (1.1 g ai ha^{-1}), thiophanate-methyl (1.2 g ai ha^{-1}), and captafol (1.7 g ai ha^{-1}) to winter wheat (cv. Maris Ranger) led to a significant reduction in the total number of colonies of *Sporobolomyces* spp. and *Cladosporium* spp. observed from leaf imprints on PDA, to 1.8, 7.8 and 4.4 % leaf area covered, respectively for each fungicide, compared with 36.2 % for the untreated leaves. The number of colonies of *Cladosporium spp.* were also significantly decreased by these fungicides from 113.9 colonies per cm^2 to 1.8, 0.3 and 15.5 for these three fungicide respectively. According to these authors, *Cladosporium* spp. were very sensitive to the fungicides applied which relates to observations presented here. In contrast, Bainbridge and Dickinson (1972) using a nail varnish impression method where an impression of the leaf surface and spores is made for identification purposes, found that the number of colonies of *Cladosporium* spp. recorded on the adaxial surface of leaves were increased from 19 (per 8 mm^2) for untreated leaves to 27 and 32 for those treated with captafol and maneb plus fentin acetate respectively. However, on the abaxial surface, the number of colonies recorded was reduced from 42 for the untreated leaves to 15 and 26 for the two fungicides, respectively. The number of *Alternaria* colonies recorded for untreated leaves was 3 on the adaxial surface and 1 on the abaxial surface, whereas it was 7 and 0 respectively for captafol treated leaves and 3 and 12 respectively for maneb plus fentin acetate treated leaves. The number of yeasts was decreased on both leaf surfaces for both fungicides.

pathogens, saprophytic microflora and fungicides. The reduction in mycelial growth of the pathogens *F. culmorum* and *M. nivale* due to antagonism by *A. alternata, B. cinerea* and *C. herbarum* depended on the sensitivity of the three saprophytic microflora species to the fungicides. The fungicides prochloraz and tebuconazole caused a reduction in the diameter of *F. culmorum* and *M. nivale* whether the microflora species were present or not, however, in the presence of chlorothalonil and pyrimethanil, the relationships between microflora and pathogens were less clear. For example, *F. culmorum* was shown to be tolerant of pyrimethanil (chapter 3), however *B. cinerea* was sensitive to this fungicide. Hence, the increased growth of *F. culmorum* on pyrimethanil-amended media in the presence of *B. cinerea* may be explained by a reduction in the growth saprophyte leading to increased colony diameter of the pathogen. Similarly, the reduction of growth of *C. herbarum* by tebuconazole and chlorothalonil at 2 μg ml^{-1} may explain the increased colony diameter of *M. nivale.* It is possible that on wheat ears, saprophytes such as *A. alternata*, *B. cinerea* and *C. herbarum* and the facultative pathogen *F. culmorum* may be competing for the same food source; applications of some fungicides may preferentially inhibit the saprophytes, allowing the niche to become occupied by the 'pathogen'. For example, Wainwright *et al.* (1992) found that applications of tebuconazole caused a reduction in the spore counts of *Cladosporium* spp., *Alternaria* spp., and *Botrytis cinerea* on the leaves and ears of wheat. Preliminary studies *in vitro* indicated the differential effects of fungicides on saprophytes and ear blight pathogens *F. culmorum* and *M. nivale*. Fungicides which reduce the mycelial growth of microflora that normally produce antibiotics inhibitory to the pathogen, may reduce their antagonistic ability and therefore allow pathogen establishment. It is, therefore, possible that application of some fungicides increases the potential for FEB, by decreasing the populations of antagonistic microflora species. The poor performance of fungicides in the field may be attributed to a reduction in competitive microflora, however, this is yet to be confirmed by further studies *in vivo.*

CHAPTER 7

The Effect of Time of Application, Cultivar and Fungicide on the Retention of Sodium Fluoroscein Tracer on the Ears of Winter Wheat and a Study of the Retention, Penetration and Metabolism of Prochloraz Using Radio-labelled Fungicide

Introduction

Deposition and retention studies for pesticides have centred mainly on the efficiency of the spray application process and on formulation studies to determine whether adjuvants (particularly surfactants) improve the retention of agrochemicals. Experiments have involved the application of fluorescent tracers using various applicators at a number of different application rates and pressures to crop and weed species to determine the efficiency of the application process. For example, Pereira (1967) appraised the use of fluorescent tracers to assess spray efficiency in the control of coffee berry disease.

Grayson and McCarthy (1987) analysed spectrophotometrically washings taken from winter wheat (cv. Fenman) plant parts sprayed with Gentian Violet, either in solution with water and acetone or with emulsifiers dissolved in mixed petroleum xylenes. They found that 65 % of the volume which was applied and deposited on the shoot, was deposited on the flag leaf, 22.5 % on the penultimate leaf and 12.5 % on the ear. Little if any deposition occurred below the penultimate leaf. Studies by Andersen *et al.* (1987) involved analysis of surface tension and contact angles of spray deposits on the surface of plant species to determine the effect of surfactants. Retention was determined by spectro-fluorometric analysis of sodium fluoroscein deposits. It was shown that coverage and spread increased with leaf age from 9.2 in young leaves to 17.7 and 37.1 % retention in intermediate and old leaves, respectively. The percentage plant coverage was linearly related to retention. Their work also showed that retention varied according to the cultivar sprayed because of the differential quantities of crystalline waxes. Studies on the ears of the winter wheat cultivar Avalon showed that on newly emerged ears (GS 55-59), the retention was 0.58 mg g^{-1} fresh weight compared with 0.79 mg g^{-1} at GS 65. Surfactants improved retention of the tracer in the younger ears but not in older ears. Andersen *et al.* (1987) showed that there was a decrease in surface roughness, shown microscopically,

and leaf disposition as leaves age. It is possible, therefore, that these observations can be extended to ears and help to explain why there was greater retention on ears at GS 65 than GS 55-59 in work reported by Andersen *et al.*, (1987). Taylor and Andersen (1987) also proposed that crop growth and not volume of application or drop size was important with respect to penetration of the crop. For the winter wheat cultivar Mission, they found that retention of the fluorescent dye Helios at GS 22 was 0.9 µl in 100 litres of water per ha compared to 12.2 at GS 45 and 20.6 at GS 85.

It is apparent from the literature that the method of application, plant species and leaf/ear age can influence the amount of spray deposited and retained on the ear. There are a number of commercially important cereal diseases such as sooty mould (*Alternaria* and *Cladosporium* species), Fusarium ear blight and glume blotch (*Septoria nodorum*) which are treated by fungicide applications to the ear. It is, therefore, important to understand more about the quantity and distribution of fungicides applied to the ear and whether the product can reach the site of infection of the pathogen. This work aimed to determine whether the amount of spray retained on ears of wheat was influenced by the stage of ear development, cultivar and fungicide. A radio-tracing experiment was also designed to determine where on the ear ^{14}C-prochloraz was retained and whether it penetrated the tissue or was metabolised into non-active hydrolysis products.

Materials and Methods

Fluorescent tracer studies

The fluorescent tracer sodium fluoroscein was used to determine the effect of time of application and cultivar on the retention on ears of winter wheat. Winter wheat ears were produced according to the procedure outlined in Chapter 2, page 35. Six cultivars were used

of contrasting resistance to FEB; Brigadier, Cadenza, Hussar, Mercia, Riband and Soissons. The length of rachis, number of spikelets and number of extruded anthers were measured on eight replicate ears for each cultivar at growth stages 59, 61, 65 and 70. For each cultivar, ears were sprayed with sodium fluoroscein (0.025% w/v plus 0.1%Agral surfactant w/v, Hogg Laboratory Supplies, Sloane St, Birmingham) at either GS 59, 61, 65 or 70. The fluorescent tracer was applied using a boom carrying two flat fan nozzles (110°) held 34 cm above ear height. After spraying, plants were incubated at 15°C out of direct sunlight for 20 minutes, after which time, individual ears were harvested, placed into plastic bags and washed by shaking in 20ml of sterile tap water for 15 minutes.

Each solution of fluorescent tracer obtained was then decanted into separate scintillation vials and placed into a fluorescent spectrophotometer (Perkin Elmer Luminescence Spectrophotometer LS30, UK, excitation wavelength 420nm, emission wavelength 508nm) to determine the amount of fluorescent tracer retained on individual ears. The ears were dried in an oven for 48 hours at 80°C until a constant mass was recorded. The amount of tracer deposited on the ears was expressed as the volume tracer per g dry weight of ear ($\mu l\ g^{-1}$ dry weight).

In a similar experiment, sodium fluoroscein was used to determine whether fungicide affected the amount of tracer retained on the ears of wheat. Prochloraz, chlorothalonil, pyrimethanil and tebuconazole were applied at field rate (Chapter 2, page 36) of winter wheat (cv. Avalon) at mid-anthesis. The spray solution in each case was made by adding the active ingredient to the solution of sodium fluoroscein (0.025 % w/v plus 0.1 % w/v Agral) in order that the tracer was the spray carrier. All fungicides were applied using a precision pot sprayer in the glasshouse (see Chapter 2, page 35). Plants sprayed with sodium fluoroscein in the absence of any fungicide acted as controls. The tracer was allowed to dry on the ears and then ears were

removed and tracer extracted as described above, to determine the volume tracer (µl) per g dry weight of ear. Rachis length, number of spikelets per ear and number of extruded anthers per ear were also determined.

^{14}C-prochloraz studies

A study of the retention, penetration and metabolism of prochloraz was undertaken at AgrEvo (UK) Limited, Chesterford Park. Spring wheat plants (cv. Baldus) were sown into John Innes no.2 compost in 17cm diameter plastic pots at a rate of seven plants per pot. Plants were grown outdoors. At anthesis plants were taken into the glasshouse 24 hours prior to spray application. Ears at mid-anthesis (GS 65) were labelled accordingly.

^{14}C-prochloraz was prepared by the formulations department of AgrEvo (UK) Limited. The radiochemical purity as determined by thin layer chromatography (TLC) was 91.24% and the specific activity was calculated to be 32.1 µCi mg^{-1}. Exactly 4.66 mg of ^{14}C-prochloraz was formulated as an emulsifiable concentrate (EC) using 10mg of technical (cold) prochloraz.

^{14}C-prochloraz was applied to ears at GS 65 at a rate of 0.9 l ha $^{-1}$ and 3 bar pressure using a custom built track sprayer. The sprayer was calibrated prior to use with water sensitive paper and volume measurements. Unsprayed plants served as controls. The deposit was allowed to dry on the ears for 1 hour in subdued light. Ears were removed immediately following application and again at intervals of 24 and 48 hours following treatment. Between sampling intervals, plants were placed in a cool bay of the glasshouse (20°C +/- 2°C). After the final sampling date, the remaining plant material was disposed of. Five ears constituted a sample and three replicate samples were taken at random for each time interval. Ears were immediately dissected into outer glumes, florets (including anthers) and rachis following sampling. Samples

were weighed and frozen following dissection.

The glumes, florets and rachis were homogenised separately using an 'Ultra-Turrax' homogeniser (Ultra Turrax T25, Janke and Kunkel, IKA Labortechnik, Staufen FRG). The homogenates were extracted three times for five minutes in acetone and once in acetone/ water (50:50 v/v). Volumes were measured and extracts sampled for liquid scintillation counting (LSC). Radioactive samples in solvent extracts were processed directly with Starscint scintillation cocktail (Packard Instrument Company, USA) and radioactivity was measured using a liquid scintillation counter (Beckman 5000TD + AgrEvo (UK) Ltd software). Extracts were 'cleaned' using Bond Elut Extraction Cartridges (Isolute, International Sorbant Technology Limited, Mid-Glamorgan) and concentrated using a Turbo-Vap 500 (Zymark Corporation, Massachusetts) closed cell concentrator. The concentrated extracts were collected for analysis of metabolites and radioactivity determined by liquid scintillation counting (LSC) to calculate the % recovery of ^{14}C in the clean concentrated extract.

Oxidations of extracted fibre were undertaken to determine the amount of prochloraz bound in fibres of glume, floret and rachis tissue. $^{14}CO_2$ generated by a sample oxidiser (Packard Oximate 80, Pangbourne, Berks) was trapped by an absorbent (carbo-sorb, Packard, USA). The absorbent was immediately mixed with scintillation cocktail (Permafluor E, Packard, USA) and analysed using LSC.

Thin layer chromatography (TLC) was undertaken for one replicate from each treatment to determine whether prochloraz had been metabolised in the tissue. Extracts were analysed using pre-coated TLC plates (20 x 20cm SIL G-25, 0.25mm silica gel with fluorescent indicator UV254, Camlab, Cambridge). Approximately 2000 decays per minute (dpm) of the

concentrated, clean extracts plus cold standards were spotted onto TLC plates and allowed to dry. The plates were placed in TLC tanks containing the solvent system; ethyl acetate: hexane: glacial acetic acid (50:50:1) and run for 45 minutes. The plates were removed and allowed to dry in a fume cupboard. The locations of compounds and standards of prochloraz and two known metabolites (BTS44595 and BTS44596) were made apparent by inspection under UV light. The detection and quantification were carried out using a Radio-TLC Linear Analyser.

Total radioactivity was determined for each plant part as mg kg^{-1} calculated from the results of LSC for extracts and oxidation of fibres. Data were transformed and analysed using analysis of variance (Genstat 5 Version 3.1, Lawes Agricultural Trust, Rothamsted, Herts).

Results

Sodium fluoroscein tracer studies

Analysis of variance for the log transformed data from the spray retention on different cultivars showed no significant difference between replicates. However, there was a significant ($p<0.05$) difference between varieties in terms of the amount of tracer retained on ears. Also, the stage of plant development (GS 59, 61, 65 or 70) influenced the amount deposited as did the interaction between variety and time, indicating that the effects of variety and time of application were both significant but did not act independently (Figure 22). Significantly greater amounts of tracer were retained on the ears of Soissons and Brigadier than the other four varieties.

Analysis of variance of regression showed that no significant correlations between the amount of tracer retained per gram dry weight of ear ($\mu l\ g^{-1}$ DW), the length of rachis or the number of spikelets. There was a significant relationship between rachis length and the amount of tracer

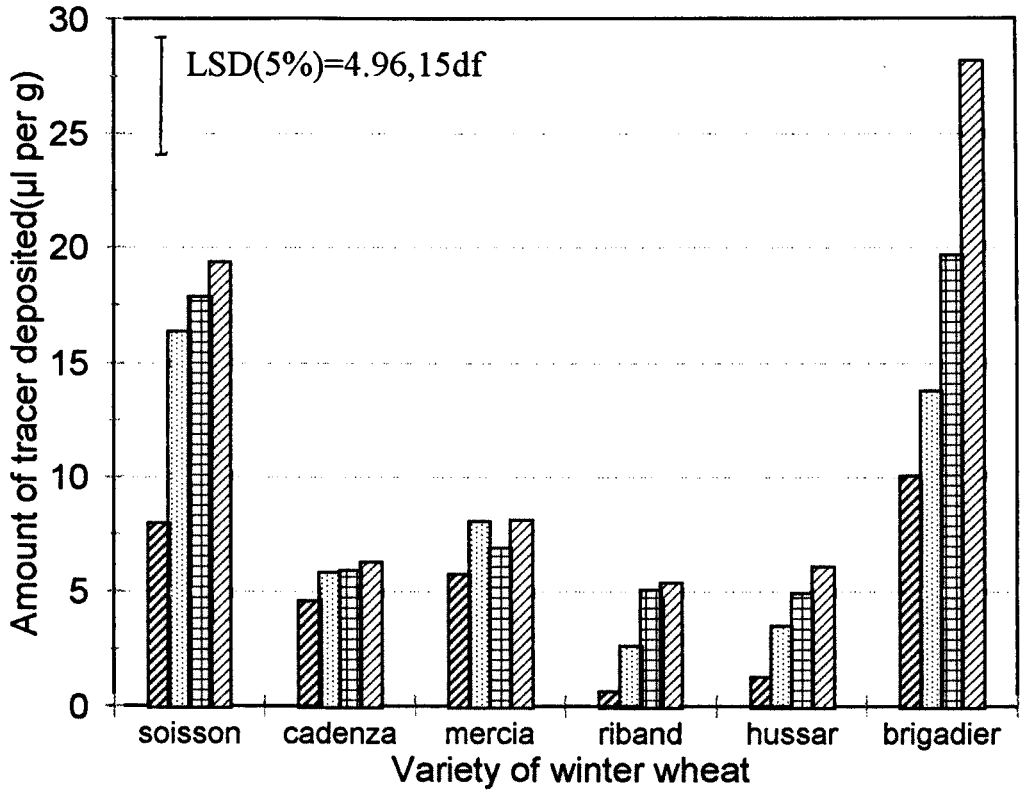


Figure 22. Retention of sodium fluoroscein (µl g-1 dry weight of ear) on ears of six winter wheat cultivars following application of sodium fluoroscein in 210l of water at growth stages 59 (▨), 61 (▦), 65 (▦) and 70 (▨). The LSD bar represents the LSD for cultivar/timing interactions.

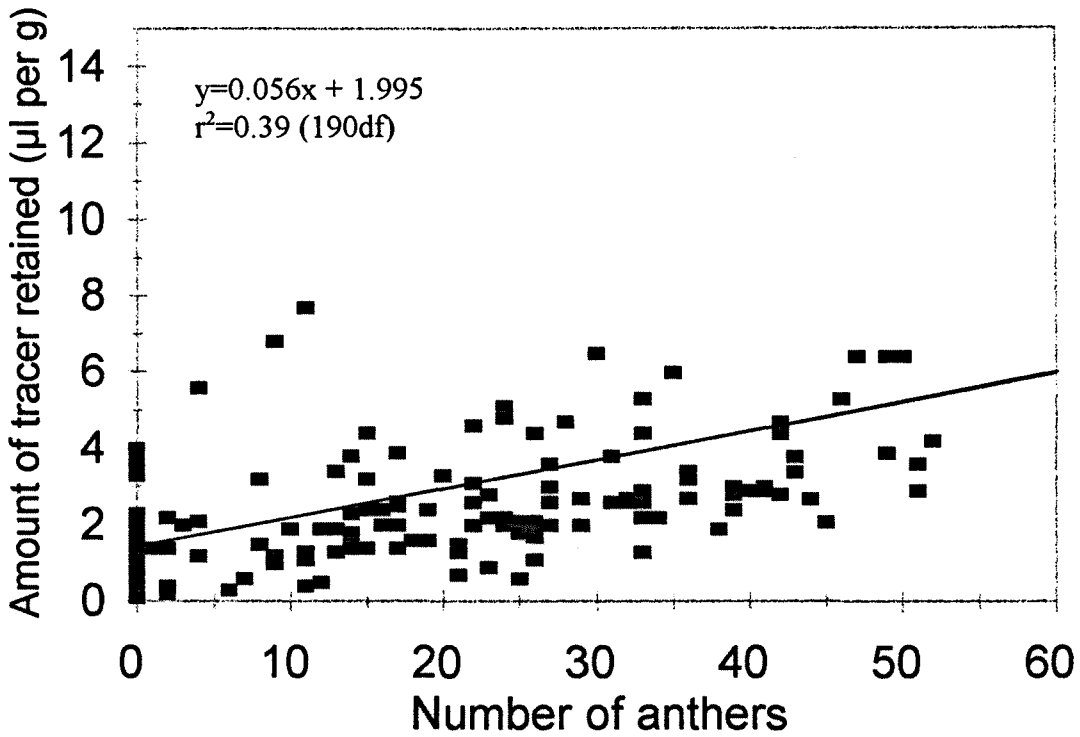


Figure 23. Relationship between the amount of sodium fluoroscein (µl g^{-1} dry weight) retained on wheat ears and the total number of anthers extruded during anthesis for six cultivars of winter wheat

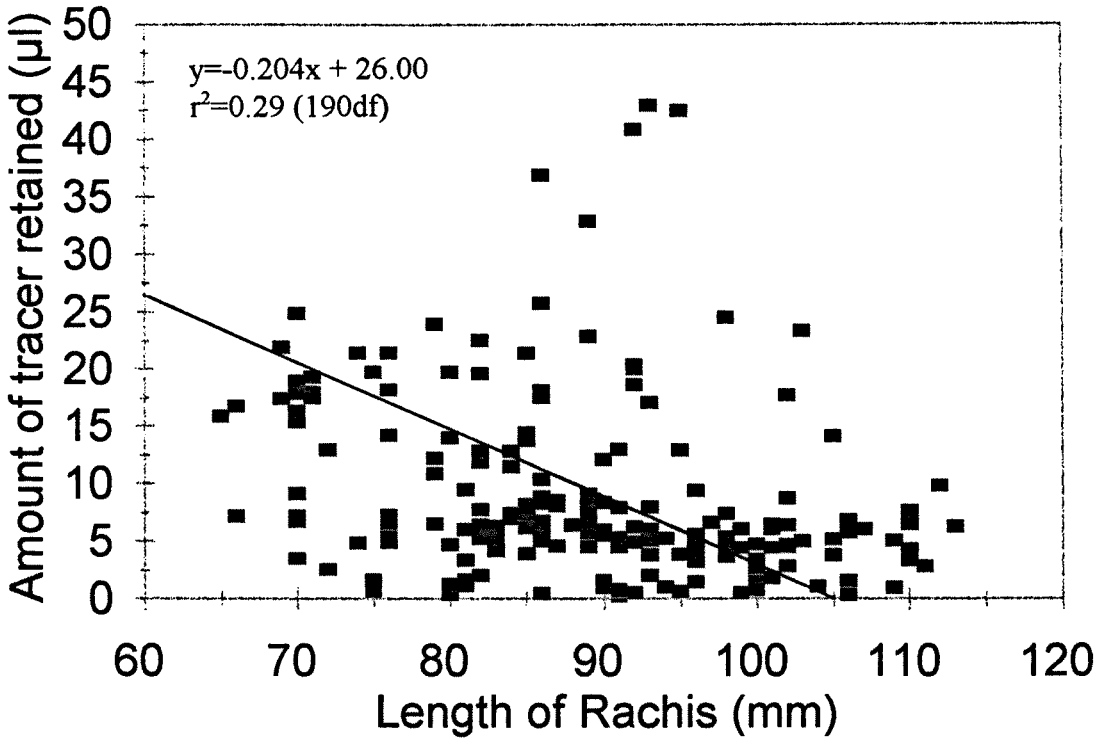


Figure 24. Relationship between the amount of sodium fluoroscein (µl) retained on wheat ears and the length of rachis for six varieties of winter wheat.

(µl). Figure 23 shows the relationship between the number of anthers present and amount of tracer per gram dry weight of ear retained following regression analysis. A significant relationship between these characteristics ($p<0.001$) was apparent with 39 % of the variance accounted for. A significant relationship was also determined between rachis length and the amount of tracer in µl ($p<0.001$)(Figure 24).

Analysis of variance of the amount of sodium fluoroscein (µl) retained on the ears of Avalon in the presence of fungicides is shown in Figure 25. Data showed that significantly greater amounts of tracer were retained when ears were treated with tebuconazole than when treated with either prochloraz, pyrimethanil or chlorothalonil. For example, when applied pre-anthesis, tebuconazole increased the retention of sodium fluoroscein from approximately 15 µl to 17.5 which compares with only 4.72, 2.22 and 2.60 µl for prochloraz, pyrimethanil and chlorothalonil respectively. The amount retained on ears with tebuconazole was greater than for the sodium fluoroscein control ears (no fungicide present) although this was not statistically significant ($p<0.05$) when applied mid-anthesis. There was significantly less retention of sodium fluoroscein on ears treated with prochloraz, pyrimethanil or chlorothalonil, compared to controls. For all fungicides, there were significantly greater amounts of sodium fluoroscein retained on ears at mid-anthesis, compared to ears at pre-anthesis.

^{14}C-prochloraz studies

For the ^{14}C-prochloraz location studies, the extraction, clean-up and concentration procedures gave efficient recovery of radioactivity from the tissue. (See Table 21).

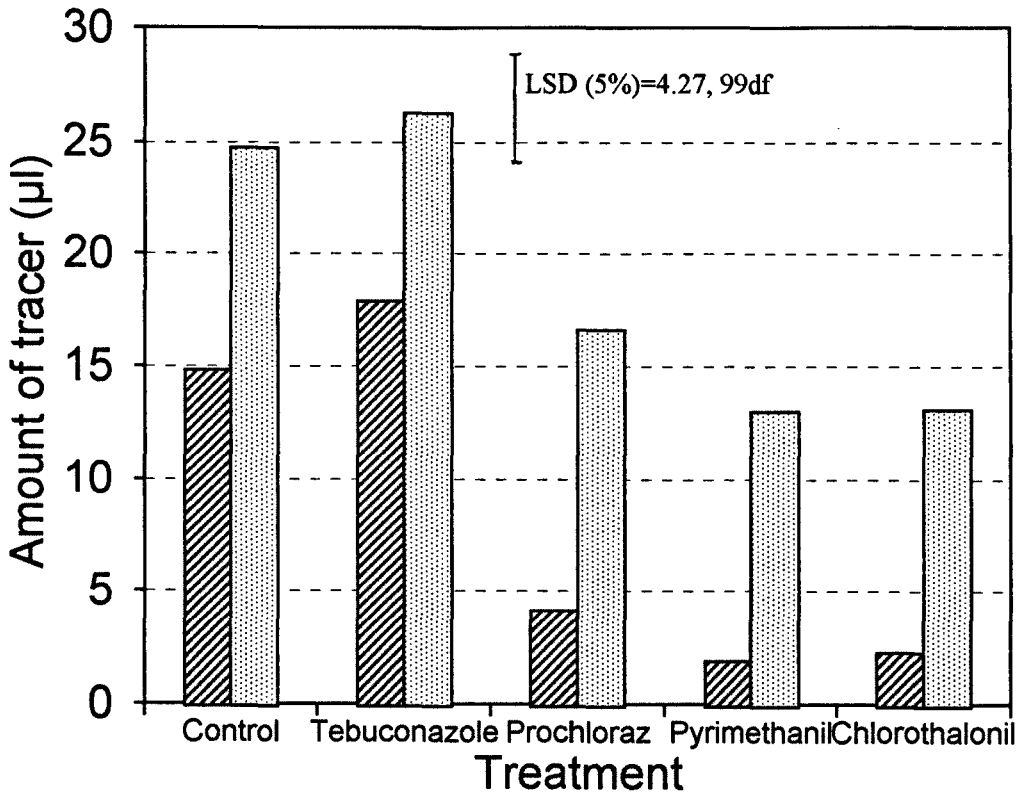


Figure 25. Retention of sodium fluoroscein (µl) on ears of winter wheat (cv. Avalon) following application of 4 fungicides at growth stages 59 (▨) and 65 (▯) compared to retention on control plants where no fungicide was applied. The LSD bar represents the LSD for fungicide/timing interactions.

Table 21. Recovery of radioactivity from plants treated with ^{14}C-prochloraz

Time Interval	% ^{14}C Recovered Following Procedure					
	Extraction	Stdev	Clean-Up	se	Conc	se
0	96.35	6.08	86.60[a]	21.96	94.60	5.11
24 h	94.50	1.76	93.99	1.70	97.18	1.23
48 h	90.37	3.97	94.75	1.58	96.39	0.68

[a] some loss in recovery due to spillage of one replicate sample

The mean decays per minute (dpm) on the glumes, florets and rachis at the 3 time intervals is shown in Table 22 and the actual amount of ^{14}C extracted from a single ear is shown in Table 23.

Table 22. Decays per minute in the glumes, florets and rachis of ears treated with ^{14}C-prochloraz immediately following spray application, 24 hours following and 48 hours following application.

Treatments	Plant Part	Mean dpm	se
Control	glumes	5727	9693
	florets	8334	10126
	rachis	3977	5521
Time=0	glumes	736267	200997
	florets	1061233	511128
	rachis	232997	118876
Time=24 h	glumes	784137	214352
	florets	1028460	290503
	rachis	227372	59353
Time=48 h	glumes	746046	446161
	florets	758040	309850
	rachis	176644	86337

The dpm data suggests that retention on the florets was greater than on glumes and rachis. However, the total dpm does not take into account the amount of material from which the ^{14}C was extracted. There were greater amounts of material for the floret samples than for the glumes or rachis. Hence the data for total dpm was manipulated to give mg of ^{14}C per kg of plant material to make the data comparable (Figure 26).

Table 23. The mean amount of ^{14}C (dpm) recovered from a single ear

Treatment	Ear Part	mean dpm	mean wt (g)	% of total dpm
Control	glumes	1145	0.159	*
	florets	1667	0.599	*
	rachis	795	0.149	*
t=0	glumes	147253	0.146	36.26
	florets	212247	0.586	52.26
	rachis	46599	0.131	11.47
t=24h	glumes	156827	0.161	38.44
	florets	205692	0.602	50.42
	rachis	45474	0.126	11.14
t=48h	glumes	149209	0.137	44.39
	florets	151608	0.524	45.10
	rachis	35329	0.124	10.51

*Radioactivity recorded for control (untreated) ears was attributed to contaminated equipment and was at too low a level to be characterised to confirm that it was not prochloraz or its associated metabolites.

Analysis of variance for the mg of ^{14}C per kg of plant material data showed that there was no significant difference between replicates, however, the main effects of plant part and time following prochloraz application were significant ($p<0.05$). Significantly greater amounts of ^{14}C

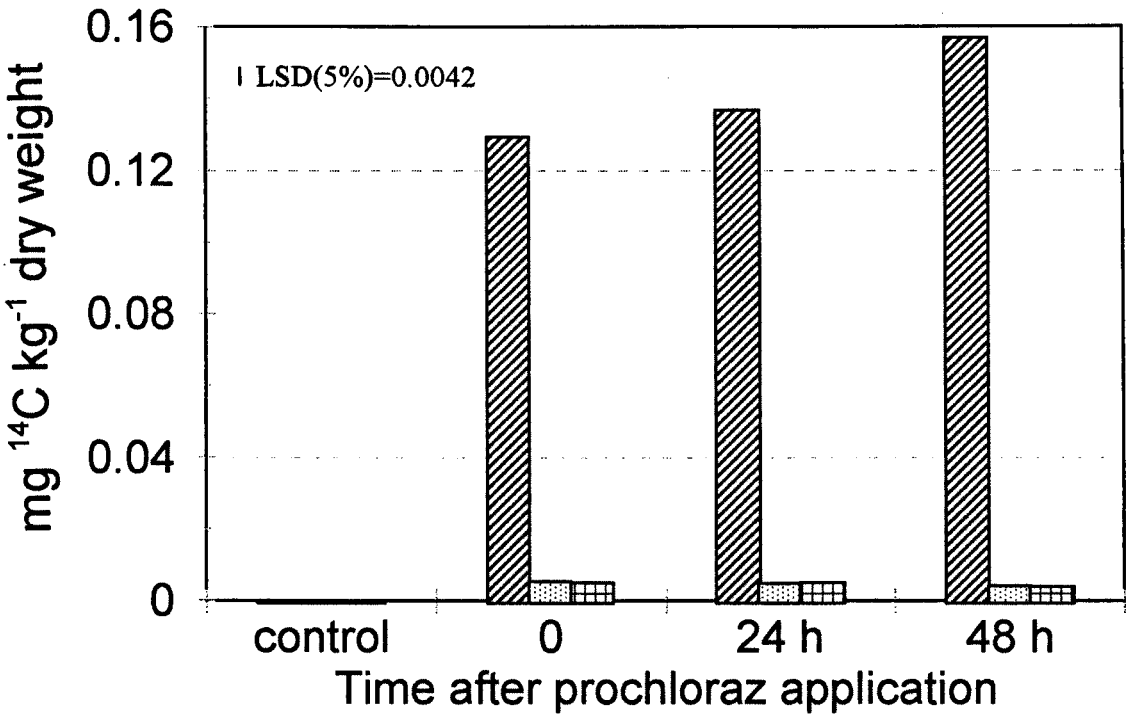


Figure 26. The effect of time following application of ^{14}C-prochloraz on the amount of ^{14}C (mg per kg) recovered from the glumes (▨), florets (▩) and rachis (▦) of spring wheat cultivar Baldus.

were measured on the glumes than the florets or rachis. For example, 24 hours after application, the glumes contained 0.01367 mg ^{14}C compared to 0.00473 and 0.00507 for the florets and rachis, respectively. There was also a significant increase in the amount of ^{14}C retained on the glumes between t=0 and t=48 hours from 0.01293 to 0.01567 mg ^{14}C. There was no significant difference between the other ear components over the 3 time intervals (Figure 26).

The mean recovery of prochloraz and its metabolites as a result of the TLC characterisation are shown in Table 24. Analysis of variance for the TLC data showed that there were no significant differences between the percentage of prochloraz or its metabolites accounted for in the glumes, florets or rachis. There was hence no reason to suggest that metabolism of prochloraz was greater in any particular ear component over a 48 hour period. However, there were significantly less of the 'unknown 1' metabolite at 48 hours than time zero for all ear components. There was also a significantly greater accumulation of polar conjugates at the origin after 24 and 48 hours compared to time zero.

Table 24. Characterisation of ^{14}C extracted from glumes, florets and rachis of spring wheat (cv. Baldus) following application of ^{14}C-prochloraz

Data as percentage values

time after treatment	plant part	prochloraz	bts44595	bts44596	unknown1	unknown2	origin	remainder
0	glumes	75.46	0	11.68	5.03	0	4.19	3.63
24h	glumes	58.46	0	24.11	3.85	0	11.36	2.21
48h	glumes	51.39	0	24.57	3.89	0	18.50	1.64
0	florets	66.22	0	17.51	5.41	0	7.88	2.98
24h	florets	62.14	0	16.96	4.58	0	12.61	3.71
48h	florets	47.93	0	22.86	3.94	2.27	16.40	6.59
0	rachis	36.48	0	46.35	5.01	0	6.26	5.90
24h	rachis	75.99	0	10.21	4.63	0	7.40	1.77
48h	rachis	59.30	0	20.57	3.11	0	13.97	3.04

Hydrolysis products of prochloraz;
bts44595 - N- propyl - N- [2 - (2, 4, 6 - trichlorophenoxy) ethyl] urea
bts44596 - N - formyl - N' - propyl - N - [2 - (2, 4, 6 - trichlorophenoxy) ethyl] urea

'Origin' - represent the immobile polar conjugates accumulated at the site of application of the samples on the TLC plate

Discussion

The tracer experiment employed a useful technique for the quantification of product applied to the ears. Varieties differed significantly in the amount of tracer deposited on the ear, although the general trend was that larger amounts of tracer were retained on ears towards the end of anthesis compared to pre-anthesis. This may imply that as anthesis progresses and the florets become more open allowing the anthers to extrude, maximum retention of tracer occurs. The study of floret and anther morphology and distribution would help to clarify this further. This increased retention agrees with Andersen *et al.* (1987) who showed that the amount of sodium fluoroscein tracer was significantly greater on ears at GS65 compared with ears at GS 55-59. According to Andersen *et al.* (1987), this increase may have been associated with decreased surface roughness on the ears as they develop which was observed on leaves. Further work is required to investigate this suggestion. In this experiment, the amount deposited per gram dry weight of ear ($\mu l\ g^{-1}$ dry weight) was correlated with the number of extruded anthers and the amount (μl) was correlated with rachis length. There were no correlations with the number of spikelets. Although this implies that maximum deposition of fungicide can occur at the end of anthesis, this would probably be after infection had occurred at the susceptible stage of plant development (early to mid anthesis).

The difference in retention of sodium fluoroscein tracer on different cultivars occurred for several possible reasons. It is possible that the characters of gross ear morphology may affect the inherent wettability of different cultivars and the type and distribution of surface waxes, roughness characteristics and the presence of trichomes may be responsible for these differences. A study of cultivar differences in terms of ear morphology would provide useful information as to why cultivars differ in their susceptibility to infection by specific pathogens and why they differ in their retention of fungicides which may contribute to their differences in

susceptibility to infection. There was no evidence of variety differences in cuticle and wax deposition with respect to ear morphology in the work reported here, however, there is evidence in the literature for differences between species which cause differences in retention. Ruiter de *et al.* (1990) showed that the retention by leaves of *Solanum nigrum, Chamomilla recutita, Elytrigia repens, Triticum aestivum, Pisum sativum* and *Solanum lycopersicum* was significantly different due to differences in crystalline epicuticular waxes and trichomes. In cultivar trials at the National Institute of Agricultural Botany (Jachman and Pickett, 1992), the morphology of cereal ears is recorded by assessments of spikelet density and ear shape and whether there are awns, scurs or hairiness of the rachis. For example, the cultivar Soissons is recorded with ears of medium length, medium density of spikelets, a tapering shape and awns along its entire length. Hussar and Cadenza are both medium length, have a lax to medium density of spikelets, a tapering shape and scurs. In contrast, Riband has a medium length, medium to dense number of spikelets and is parallel in shape, bearing scurs on the top quarter of the ear. No quantitative details of the characters are given and the description merely gives an overview of ear morphology. Further quantitative research would be required to relate ear morphology and differences in cuticle between cultivars with retention. Such studies may be useful breeding characteristics in order to optimise fungicide retention.

The fungicide retention studies showed that the sodium fluoroscein technique could be applied to study the retention of fungicides on ears of wheat. There were differences between the retention of the four fungicides. All four products tested are currently formulated for foliar application, hence the adjuvants used in these formulations are designed specifically for application to foliage. Due to the inherent differences between the cuticle and wax morphology of leaves and ears (Andersen *et al.*, 1987), it may be that fungicides should be formulated specifically for application to ears. In the case of prochloraz, pyrimethanil and chlorothalonil,

the retention of the formulated product (with the surfactant Agral in the spray solution) was significantly lower than for the tracer plus Agral alone. This suggests that the addition of the non-ionic wetter/spreader Agral resulted in greater retention of the tracer to ears than the formulated products which contained alternative surfactants in addition to the Agral. These results show that the application of fungicides to the ears of wheat warrants considerable research to analyse the fungicide/adjuvant combinations which optimise the retention and penetration of the fungicide active ingredient. A limitation of the sodium fluoroscein technique was that sodium fluoroscein was carried in the aqueous phase of the spray solution and not necessarily with the active ingredient if it was present in the oil phase of an emulsion, hence the experiment should be repeated using tracers such as TP 104 (Williams Ltd, Hounslow, Middx) which is an oil-soluble fluorescent dye soluble in acetone where appropriate. One further limitation is that this technique quantifies tracer on the whole ear and not the components of florets, rachis and outer glumes, because the technique does not allow plant dissection without some contamination between plant parts. It would be useful to quantify the site of deposition and retention, hence, this could be correlated to the site of infection.

The radio tracing experiment indicated the location of prochloraz following application, since the extraction, clean-up and concentration procedures were efficient. The data gives an accurate estimate of the location of prochloraz and its metabolites. There was no difference between the amounts of ^{14}C recovered from the glumes, florets or rachis with time following ^{14}C-prochloraz application, suggesting that the ^{14}C-prochloraz did not penetrate the glumes and was not translocated away from the site of deposition. Hence, there was no systemic movement of prochloraz. Birchmore *et al.* (1977) also showed that prochloraz lacked systemicity when compared to benomyl. The systemic uptake by roots when tested against barley powdery mildew was recorded as 10 % for prochloraz and 100 % for benomyl at an application rate of

500 ppm active ingredient.

The location of ^{14}C was predominantly on the glumes, although an appreciable amount was retained on the florets and a small amount was retained on the rachis. This was anticipated since the outer glumes enclose the reproductive parts (florets) of the ear and form the outer surface of the ear. The results obtained for the TLC rachis extract at time zero was somewhat of an anomaly. Ideally the TLC procedure should be repeated using an alternative replicate. It is important that some product reaches the florets, since infection is greatest at anthesis when the ears are particularly susceptible to the pathogen. It is likely that the reproductive parts of the ear (anthers) are the site of infection of the pathogen, however, further work is required to investigate this. It is important to highlight that ^{14}C-prochloraz was retained mainly on the florets and glumes of ears and very little appears to reach the rachis where the pathogen is predominantly found. Research using Polymerase Chain Reaction (PCR) analysis of dissected infected ears harvested from an inoculated field trial in 1994 (Doohan, pers comm), showed that 40 % or rachis samples were contaminated with *F.culmorum*, 6 % with *F.avenaceum*, 36 % with *Microdochium nivale* var. *nivale* and 18 % with *M. nivale* var. majus. This work clearly shows that fungicides are retained mainly on the outer glumes of wheat, however, the PCR evidence shows that the rachis becomes heavily colonised by the pathogen, particularly in the case of *F. culmorum.* Hence, this discrepancy between the site of fungicide retention and the site of infection and colonisation of the ear by the pathogen may explain why fungicides are not particularly effective in controlling FEB.

The evidence of metabolism of the parent compound within 48 hours of ^{14}C-prochloraz application was expected. The parent was metabolised by hydrolysis in the tissue to the primary hydrolysis product bts44596. Prochloraz ($C_{15}H_{16}C_{l3}N_3O_2$) is normally cleaved at the imidazole

ring to produce the primary plant metabolite N-formyl-N'-propyl-N-[2-(2, 4, 6-trichlorophenoxy) ethyl]urea ($C_{13}H_{14}C_{l3}N_2O_3$) which is then degraded to N-propyl-N-[2-(2, 4, 6-trichlorophenoxy) ethyl]urea ($C_{12}H_{14}C_{l3}N_2O_2$) in free and conjugated forms (Tomlin, 1995). Other metabolites include 2-(2, 4, 6-trichlorophenoxy)ethanol, 2-(2, 4, 6-trichlorophenoxy) acetic acid and 2, 4, 6-trichlorophenol and conjugates of the above.

The presence of 'unknown 1' was probably due to the impurity of the ^{14}C-prochloraz applied and was not significantly different for any plant part at any time interval. The decrease in parent compound with time was also associated with an increase in the accumulation of polar conjugated material at the origin. Further work would investigate the nature of the polar conjugates at the origin and the effect of a change in application volume, pressure and product formulation on the deposition and retention of prochloraz (and other fungicides) on the ears of wheat.

These experiments indicate that fungicide retention on the ears of wheat can vary enormously depending on the plant growth stage, the fungicide used and the cultivar treated. There are many other factors which influence fungicide retention and ultimately their efficacy. For example, fungicide performance may be limited by the environment; the volatility, hydrolysis, photolysis, wash-off, evaporation and drift of products may influence the fungicide active ingredient actually reaching the site of activity (Steurbaut, 1993). Many of these factors have been used to explain the discrepancies between the glasshouse and field performance of fungicides, since many of these factors are minimised under glasshouse conditions (Gold *et al.*, 1994). In addition, the adsorption, absorption, penetration, distribution and translocation of fungicides is influenced not only by the environmental conditions, but also by the nature of the fungicide and in particular its systemicity and formulation (Steurbaut, 1993). It is possible that

further studies on the performance of fungicides under a range of environmental conditions using different formulations may assist in devising recommendations for the rational application of products to prevent FEB epidemics. Clearly, the study of fungicide performance and formulation would clarify the requirement for fungicide products which are both resistant to adverse environmental factors on the ears of cereals, while being active against ear blight pathogens.

CHAPTER 8

General Discussion

Fusarium ear blight is of economic importance because it can reduce yield (Wong *et al.*, 1992), cause mycotoxin contamination of grain for feed (Joffe, 1978) and reduce seed quality (Reeves and Wray 1994). A review of the literature has shown that control of FEB by fungicide sprays has, in general, been poor and there is a discrepancy between the performance of fungicides *in vitro* and in the field. This discrepancy between performance *in vitro* and *in vivo* may be explained by several factors including application technique and formulation of products. The presence of saprophytic fungi such as *Alternaria alternata, Botrytis cinerea, Cladosporium herbarum, Epicoccum nigrum* and *Aspergillus niger* which have been isolated from wheat ears (Grabarkiewicz-Szczęsna *et al.*, 1989) may also influence field performance of fungicides. There is little experimental evidence which demonstrates that interactions between saprophytic microflora and ear blight pathogens could account for the poor performance of fungicides *in vivo*. Similarly, few studies have been undertaken to study the retention of fungicides on the ears of wheat. The aim of this research was to evaluate the efficacy of fungicides against FEB, the role of *A.alternata, B.cinerea* and *C.herbarum* in the development of Fusarium ear blight and to examine the retention of fungicides on the ears of wheat.

It was shown that the triazole fungicide tebuconazole and the imidazole fungicide prochloraz were consistently effective in reducing the mycelial growth of *Fusarium culmorum, F. avenaceum, F. poae, F. graminearum* and *Microdochium nivale in vitro*. For example, tebuconazole and prochloraz reduced the diameter of mycelial colonies of many isolates by over 90 % at concentrations of 2 $\mu g\ ml^{-1}$. In addition, the dithiocarbamate fungicide chlorothalonil completely inhibited spore germination of all species. The triazole fungicides flusilazole and tebuconazole were considerably more effective than flutriafol and fluquinconazole, despite having the same mode of action and this shows that fungicides vary in their activity against these pathogens even within the same chemical group. The tolerance of certain isolates of *M.*

nivale to benomyl *in vitro*, substantiated the evidence provided by Locke *et al.* (1987), who concluded that there was little justification for the use of benomyl to control Fusarium diseases of cereals. The fungicides tebuconazole, prochloraz, chlorothalonil and pyrimethanil were selected for further studies on the basis of their efficacy *in vitro*, however, it is possible that fungicides which may have been efficacious *in vivo*, but not *in vitro*, were not selected, therefore their activity *in vivo* was not realised. This is an obvious limitation of relying on the results of preliminary *in vitro* investigations alone, since the extrapolation of data from *in vitro* experiments to field performance, depends on not only the fungicide ED_{50} value and the slope of the dose-response curve, but also the ability to build up residues, withstand wash-off, the rate of chemical degradation and the fungal toxicity of breakdown products (Rich *et al.*, 1953).

The efficacy and timing experiments in the glasshouse and field showed that for the pathogens *F. culmorum* and *M. nivale*, the fungicides tebuconazole and prochloraz were consistently effective in reducing FEB, whereas chlorothalonil and pyrimethanil were less effective. Pyrimethanil showed some reduction in FEB caused by *M. nivale*, but it was less effective against *F. culmorum*. These results broadly compare with the results of the *in vitro* experiments in that, of the four most efficacious products *in vitro* (prochloraz, tebuconazole, pyrimethanil and chlorothalonil), prochloraz and tebuconazole were consistently the best products in reducing FEB, although pyrimethanil also showed activity against *M. nivale*.

Although all fungicide treatments significantly reduced the severity of FEB under field conditions, the fungicides were less effective than under glasshouse conditions. When the incidence and severity of FEB was recorded at GS 85, the most efficacious product in 1995 was the two spray application of tebuconazole two days before inoculation and prochloraz two days after inoculation. Although not statistically significant, pre-inoculation treatments were

in general more effective than the post-inoculation treatments. For most fungicides, the reduction in the severity of FEB became less significant with time as the season progressed and the severity of FEB increased. However, when prochloraz was applied two days after inoculation, there was no reduction in the control of the disease with time, which suggests that this fungicide retained its activity longer than the other products. This may be because prochloraz, unlike the other three products, was formulated as an emulsifiable concentrate (EC) as opposed to a suspension concentrate (SC). It was also interesting to note that there was no benefit of mixing prochloraz and tebuconazole, since the activity of the mixture was inferior to the activity of the single active ingredients, showing that there was no synergism between the two products when mixed in these proportions. However, further investigations mixing the active ingredients in different proportions may highlight synergism between the products and improved control of the disease.

In the trial in 1996, all fungicides significantly reduced the severity of FEB, although there were no significant differences between timings. In general, pyrimethanil and the copper-prochloraz formulation were slightly more effective when applied after inoculation and the fungicides tebuconazole and prochloraz were more effective when applied before inoculation which agreed with the results from the trial in 1995. The copper-prochloraz complex behaved consistently well, irrespective of timing and was more effective than the prochloraz formulation without the metal complex, suggesting that the copper component contributed to the activity of prochloraz against FEB. It was also proposed that application timing was less critical for tebuconazole, a fungicide which is systemic in plant tissue (Hassall, 1990). However, for the weakly systemic products; pyrimethanil and prochloraz, timing would be more critical with respect to pathogen infection. The systemicity of these fungicides in the ears of wheat needs to be compared in order to confirm this hypothesis.

As the season progressed, many fungicides ceased to cause an effective reduction in the severity of FEB. This may mean that under severe disease pressure, the fungicides were no longer effective, particularly when applied in advance of 48 hours prior to infection. This confirms the need to predict epidemics where possible, so that fungicides can be applied at a suitably short interval prior to an infection event. At present, the prediction of FEB is not precise, since it depends mainly on the ability to predict weather parameters, such as temperature, relative humidity and rainfall, (Jenkinson, 1994) which affect the production, dissemination and infectivity of conidia or ascospores. This in turn means that further investigations into the epidemiology of this disease are required, such as an understanding of potential inoculum sources, mechanisms of dispersal and factors affecting infection of cereal ears when the inoculum is present. It is also likely, that those conditions which are required for the production and dispersal of spores are the same conditions which limit the available spray days for the application of ear washes. For example, rainfall has been associated with an increased incidence of FEB (Sutton, 1992) and overhead irrigation has been used to increase the severity of FEB (Strausbaugh and Maloy, 1986). However, these conditions would be unsuitable for the application of fungicides, since the potential for wash-off would be increased, such as that shown for prochloraz (Cooke *et al.*, 1989).

The field-applied fungicides gave no significant relationships between the incidence and severity of disease and the yield or thousand grain weight. Due to the drought conditions of 1995 and the wet weather which caused lodging in 1996, it may be that an effect of fungicides on reducing FEB and causing a subsequent increase in yield were not apparent. It is possible that a reduction in the severity of FEB due to fungicide application was not necessarily associated with a yield increase. Further trials to assess fungicide efficacy and yield would confirm whether fungicide application can increase yield by reducing the severity of FEB.

The discrepancy between *in vitro*, glasshouse and field performance of fungicides was not unexpected. In the glasshouse, fungicide performance was variable. In the field, there are examples of fungicide application which have no effect on FEB. For example, Milus and Parsons (1994) showed that applications of tebuconazole failed to reduce the incidence of FEB caused by *F. graminearum* or increase yield. In the work presented here, there was a discrepancy between *in vitro*, glasshouse and field performance of fungicides. For example, tebuconazole reduced the mycelial growth of *F. culmorum* by 93 % at 2 $\mu g\ ml^{-1}$ *in vitro*, yet it only reduced the severity of FEB by up to 83 % in the glasshouse and up to 40 % in the field. Fungicide efficacy in the field is influenced by environmental conditions, which are usually controlled under glasshouse conditions. For example, Gisi *et al.* (1988) showed that the triazole fungicides tebuconazole, flusilazole and flutriafol were less active under cool, humid conditions, however, prolonged humid conditions are conducive to infection of ears by *Fusarium* spp. (Pugh *et al.*, 1933; Andersen, 1948). Weather conditions following spray application are important in terms of run-off, wash-off and photo-degradation of the product which may result in the redistribution of fungicide active ingredients away from the site of infection. For example, prochloraz has been shown to be redistributed on wheat following simulated rainfall and the amount redistributed is correlated with the amount of rainfall (Cooke *et al.*, 1989). In conclusion, attempts to extrapolate the performance of fungicides under artificial *in vitro* conditions to relatively controlled glasshouse conditions to the uncontrolled field conditions must be treated with caution.

In studies on the role of saprophytic microflora in the development of FEB, it was proposed that fungicides act to inhibit saprophytic microflora on the ears of wheat which are normally antagonistic to ear blight pathogens. (This is only true if the saprophytic species are more sensitive to the fungicides than the pathogens). In so doing, the niche would become available

for the pathogen to 'occupy'. In the glasshouse, *Alternaria alternata, Botrytis cinerea* and *Clad-osporium herbarum* were antagonistic to *Fusarium culmorum* when present on ears of wheat prior to the pathogen. *In vitro* studies showed that this was due, in part, to volatile and non-volatile antibiotic production. There was no reduction in the severity of FEB when the plants were inoculated with saprophytes following inoculation with *F. culmorum*, hence it seems essential for the saprophyte to become established on the ear in order to have any inhibitory effect on *F. culmorum*.

Wainwright *et al.* (1992) showed that *A. alternata, B. cinerea* and *C. herbarum* were present on the ears prior to anthesis, anthesis being the most susceptible stage of host plant development for infection by ear blight pathogens (Andersen, 1948). It is possible, therefore, that in the field situation, these saprophytes may exert a suppressive effect on the pathogen. Indeed, *Cladosporium* spp. have been shown to be antagonistic to *Cochliobolus sativus* on rye (Fokkema *et al.*, 1975) and *Botrytis cinerea* has been shown to be antagonistic to *Helminthosporium* spp. on wheat. Bateman (1979) also showed in inoculation experiments that both *Alternaria* spp. and *Cladosporium* spp. when applied to ears at mid anthesis, led to a reduced recovery of *M. nivale* from the harvested grain.

It was shown in the *in vitro* and glasshouse experiments that *C. herbarum*, *B. cinerea* and *A. alternata* vary in their sensitivity to fungicides. The results of the glasshouse experiments suggested that applications of chlorothalonil reduced the populations of all three species, whereas tebuconazole failed to reduce *B. cinerea.* All four fungicides reduced colonisation of ears by *C. herbarum*. This would mean that application of certain fungicides would reduce the populations of these species on the ears of wheat, thereby reducing their antagonism, possibly causing a subsequent increase in the severity of FEB, provided the ear blight pathogen is less

sensitive to the fungicide. Wainwright *et al.* (1992), also showed that pathogen and saprophytic microflora species were sensitive to fungicides to different extents. *Cladosporium* spp. and *Alternaria* spp., for example, were shown to be more sensitive to tebuconazole than *Fusarium* spp. Further *in vivo* studies would confirm whether the detrimental effect of fungicides on antagonistic microflora can help explain the poor performance of fungicides to control FEB.

An alternative proposal to explain the poor performance of fungicides to control FEB was that fungicides failed to reach the site of infection and colonisation by the pathogen. It was shown from PCR studies that large amounts of fungal DNA were present predominately in the rachis, and to a lesser extent, the glumes and grain (F. Doohan, pers comm, John Innes Institute, Norwich, UK). In this study, radiotracing technique confirmed that the fungicide prochloraz is retained mainly on the outer glumes. This product is non-systemic and relatively immobile in plant tissue (Birchmore *et al.*, 1977). The activity of prochloraz against FEB may be markedly improved if it was retained by the florets and rachis, sites where the pathogen has been located by PCR (F. Doohan, pers comm, John Innes Institute, Norwich, UK). Studies of systemic products such as tebuconazole and benomyl, would show whether these fungicides had more success in reaching the florets and rachis, although this is less important for mobile products which may move into the rachis. It may be that a change in the formulation of products may improve their retention and penetration, thereby improving their performance against ear blight pathogens. Indeed, rational fungicide use to control ear blight pathogens should involve studies of fungicide efficacy and their performance with respect to environmental factors which influence their ability to withstand photolysis, hydrolysis, volatility and evaporation.

These experiments showed that varieties differed in the amount of tracer deposited on ears and

maximum retention occurred at the end of anthesis. Studies by Jenkinson (1994) showed that infection of ears by *F. culmorum, F. avenaceum* and *F. poae* was optimal during early anthesis when few anthers have been extruded. This implies that fungicides should be applied at early anthesis and it would be unwise to prolong fungicide application, since infection is more likely to occur at early- to mid-anthesis. Andersen *et al.* (1987) also found that retention of tracers was increased at GS 65 compared to GS 55-59. A study of surface roughness characteristics would confirm whether this varies for different cultivars and whether a decrease in surface roughness (wax and cuticle morphology) may contribute to increased retention of tracer as ears age, as suggested by Andersen *et al.* (1987).

Proposed further studies

In this study, the effectiveness of fungicides against FEB under laboratory, glasshouse and field conditions have been highlighted. The research has gone some way to understanding the discrepancy between fungicidal control of *Fusarium* spp. *in vitro* and *in vivo*, by demonstrating the significance of spray timing, application technique and the interaction of *Fusarium* spp. with other saprophytic fungi colonising wheat ears. However, it is proposed that further studies are needed in order to clearly identify those factors which contribute to the successful chemical control of FEB in the future. Proposed further studies include:

1) *Epidemiology of FEB*

- Histological studies to show how and where infection occurs and whether this is within the florets themselves and whether infection of the rachis occurs, or whether it merely becomes colonised by the pathogen following initial infection of the florets.

- *In vitro* and *in vivo* studies of pollen. Ears may be susceptible at anthesis because pollen acts as a stimulatory substance for the pathogen, as illustrated for *Cladosporium* spp. on rye leaves

by Fokkema, (1971). It is possible that pollen is stimulatory to the extent of antagonising fungicide activity against the pathogen.

- FEB effects on yield. Further studies of inoculated and naturally infected field trials to determine the relationships between ear blight, fungicide application and yield.

- Epidemiological studies to determine the conditions conducive to spore production, dispersal and infection of ears, in order that a forecasting system be developed so that fungicides can be applied at the optimum time to prevent FEB epidemics.

- Pathogen colonisation studies. PCR studies to locate the pathogen following infection and to determine how this is altered by fungicide application. The location of the pathogen could also be determined for different cultivars and correlated with fungicide retention on the glumes, florets and rachis, to explain why certain cultivars are more susceptible to ear blight than other cultivars.

2) *Fungicide Efficacy*

- *In vitro* and *in vivo* screening to identify efficacious active ingredients and novel modes of action, taking into account the range of species which have been associated with the disease and the adaptive capabilities of the pathogen, particularly *M. nivale*, which has developed resistance to benzimidazole fungicides (Locke *et al.*, 1987).

- Analysis of secondary colonisers. Species vary in their sensitivity to fungicides, so fungicides may remove one species from the ear, allowing a more tolerant species of pathogen to colonise the ear. Experiments using controlled environment cabinets and the glasshouse may show how species interact to cause ear blight and how fungicides affect the severity of disease when more than one species is present at a time. This may suggest that a mixture of products is the best approach for controlling more than one species at a time.

3) *Fungicide Retention*

- Glasshouse and controlled environment studies to determine where fungicides are retained by ears for a range of fungicides and active ingredients in different formulations to determine whether the retention, penetration and performance is improved by altering the adjuvant combination.

4) *Mycotoxin Studies*

- Mycotoxin analysis of infected plants *in vivo*. Boyacioglu *et al.* (1992) showed that although thiabendazole failed to decrease the severity of FEB, there was a decrease in the mycotoxin (DON) contamination of the grain. Hence, *in vivo* studies to relate fungicide efficacy against ear blight and subsequent mycotoxin contamination of grain would confirm the effect of fungicides on grain quality.

- *In vitro* experiments to confirm how fungicides affect mycotoxin production by the pathogen under controlled conditions.

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Appendix 1

Media Constituents

A. Milled Wheat Agar

15g / litre technical agar
5g / litre finely milled wheat

B. Sucrose Nutrient Agar

1.0.g KH_2PO_4
1.0g KNO_3
0.5g $MgSO_4.7H_2O$
0.5g KCl
0.2g glucose
0.2g sucrose
20.0g Oxoid No. 1 bacteriological agar
1 litre distilled water

Appendix 2. Agronomy details - field trial 1995

Date	Field Operation
1/9/94	Shakeaerated to 18 " SE TO NW
4/9/94	Ploughed to 8" E to W
	Single pass with Rotera on day
27/10/94	Drilled
21/11/94	Stomp 3.3l/ha
	IPU 2.5l/ha in 231l/ha H_2O
27/11/95	Decis 150ml/ha in 300 l/ha H_2O
13/3/95	1/2 l Bas 464
	1 l Propol Mn in 240 l/ha
14/3/95	50 kg N as Nuram
20/4/95	99 kg N as Nuram
19/5/95	75 kg N as Nuram
31/5/95	Folicur 1 l/ha
12/6/95	1 l/ha Opus Plus - on Block 4
	260g/ha Aphox - on Block 4
11/7/95	Combined

Appendix 2. Agronomy details - field trial 1996

Date	Field Operation
8/11/95	Drilled @ 375 seeds/ m^2
23/11/95	Herbicide: Javelin Gold (5 l/ha)
6/5/96	Ally (30 g/ha)
6/5/96	Starane (1 l/ha)
27/5/96	Growth Regulator: Terpal (2 l/ha)
21/2/96	Nitrogen: N as Nuram (100 kg)
20/3/96	N as Nuram (60 kg)
6/5/96	Fungicide: Sanction (0.5 l/ha)
6/5/96	Mistral (1 l/ha)

Appendix 3
Yield Data - Glasshouse Trial

1. *F. culmorum* inoculated glasshouse experiment

TRT	INOC	FUNGICIDE	TIMING	No. of grains/ear	Total grain wt/ear	Mean grain wt/ear
1	N	tebuconazole	7 d pre-inoculation	14.72	0.476	0.027
2	N	chlorothalonil	7 d pre-inoculation	16.3	0.582	0.030
3	N	prochloraz	7 d pre-inoculation	18.91	0.605	0.025
4	N	pyrimethanil	7 d pre-inoculation	23.02	0.690	0.025
5	Y	tebuconazole	7 d pre-inoculation	19.4	0.707	0.031
6	Y	chlorothalonil	7 d pre-inoculation	16.98	0.448	0.024
7	Y	prochloraz	7 d pre-inoculation	16.41	0.525	0.027
8	Y	pyrimethanil	7 d pre-inoculation	16.17	0.469	0.024
9	Y	tebuconazole	2 d post inoculation	14.65	0.437	0.026
10	Y	chlorothalonil	2 d post inoculation	20.66	0.555	0.026
11	Y	prochloraz	2 d post inoculation	17.71	0.537	0.026
12	Y	pyrimethanil	2 d post inoculation	17.67	0.480	0.024
13	Y	tebuconazole	5 d post inoculation	15.12	0.426	0.021
14	Y	chlorothalonil	5 d post inoculation	13.42	0.372	0.021
15	Y	prochloraz	5 d post inoculation	14.76	0.460	0.027
16	Y	pyrimethanil	5 d post inoculation	15.73	0.445	0.024
17	Y	untreated		16.71	0.466	0.023

LSD No. of grains = 5.39
LSD Total grain wt = 0.18
LSD Mean grain weight = 0.172

Appendix 3
Yield Data - Glasshouse Trial

2. *M. nivale* inoculated glasshouse experiment

TRT	INOC	FUNGICIDE	TIMING	No. of grains/ ear	Total grain wt/ear	Mean grain wt/ear
5	Y	tebuconazole	7 d pre-inoculation	42.19	1.855	0.043
6	Y	chlorothalonil	7 d pre-inoculation	36.62	1.765	0.048
7	Y	prochloraz	7 d pre-inoculation	36.94	1.751	0.050
8	Y	pyrimethanil	7 d pre-inoculation	41.68	2.019	0.049
9	Y	tebuconazole	2 d post inoculation	31.95	1.400	0.043
10	Y	chlorothalonil	2 d post inoculation	29.83	1.300	0.044
11	Y	prochloraz	2 d post inoculation	36.48	1.636	0.043
12	Y	pyrimethanil	2 d post inoculation	30.75	1.393	0.045
13	Y	tebuconazole	5 d post inoculation	37.65	1.717	0.046
14	Y	chlorothalonil	5 d post inoculation	34.18	1.600	0.046
15	Y	prochloraz	5 d post inoculation	37.34	1.722	0.045
16	Y	pyrimethanil	5 d post inoculation	33.32	1.541	0.046
17	Y	untreated		36.04	1.578	0.042

LSD No. of grains = 6.03
LSD Total grain wt = 0.305
LSD Mean grain weight = 0.058

Appendix 3
Yield Data - Field Trial 1995

TRT	FUNGICIDE	TIMING	Yield t/ha	TGW (g)
1	prochloraz	2 d pre-inoculation	6.88	37.93
2	chlorothalonil	2 d pre-inoculation	7.07	37.37
3	tebuconazole	2 d pre-inoculation	6.91	38.14
4	prochloraz	2 d post-inoculation	7.14	38.69
5	pyrimethanil	2 d post-inoculation	6.73	38.05
6	tebuconazole	2 d post-inoculation	6.98	38.17
7	tebuconazole + prochloraz	2 d pre -and 2 days post-inoculation	6.88	37.67
8	tebuconazole + prochloraz (150/300 tank-mix)	2 d post-inoculation	6.82	38.16
9	*B. subtilis*	2 d post-inoculation	7.14	38.7
10	control		6.70	37.49

LSD Yield = 0.90
LSD TGW = 2.05

Appendix 3
Yield Data - Field Trial 1996

TRT	FUNGICIDE	TIMING	Yield t/ha	TGW (g)
1	prochloraz	7 d pre-inoculation	3.47	49.90
2	prochloraz	5 d pre-inoculation	4.19	49.85
3	prochloraz	3 d pre-inoculation	3.26	46.21
4	prochloraz	1 d pre-inoculation	4.32	47.99
5	prochloraz	1 d post-inoculation	4.54	52.75
6	pyrimethanil	7 d pre-inoculation	3.14	45.76
7	pyrimethanil	5 d pre-inoculation	4.37	46.35
8	pyrimethanil	3 d pre-inoculation	3.55	47.39
9	pyrimethanil	1 d pre-inoculation	5.49	49.13
10	pyrimethanil	1 d post-inoculation	4.32	47.34
11	tebuconazole	7 d pre-inoculation	2.85	45.63
12	tebuconazole	5 d pre-inoculation	3.74	52.56
13	tebuconazole	3 d pre-inoculation	3.87	49.51
14	tebuconazole	1 d pre-inoculation	3.33	52.71
15	tebuconazole	1 d post-inoculation	4.43	42.82
16	Cu-prochloraz	7 d pre-inoculation	4.58	52.98
17	Cu-prochloraz	5 d pre-inoculation	4.28	44.08
18	Cu-prochloraz	3 d pre-inoculation	5.58	49.78
19	Cu-prochloraz	1 d pre-inoculation	5.48	50.95
20	Cu-prochloraz	1 d post-inoculation	5.15	48.42
21	control		5.54	48.78

LSD Yield = 2.00
LSD TGW = 9.21